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UNIVERSITY OF ALBERTA

EFFECTS OF GLUTAMINE AND EXERCISE ON IMMUNE FUNCTION AND TUMOR GROWTH

by

Leann Dawn Shewchuk

©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in Nutrition and Metabolism.

Department of Food Science and Nutrition

Edmonton, Alberta

Spring 1994

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled EFFECTS OF GLUTAMINE AND EXERCISE ON IMMUNE FUNCTION AND TUMOR GROWTH submitted by Leann Dawn Shewchuk in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.

Catherine J Field, Supervisor

Vickie E Baracos

Michael I McBurney

Vickie J Harber

TO MY FATHER FOR HIS LOVE AND ENCOURAGEMENT

ABSTRACT

The effect of exercise and dietary L-glutamine supplementation on immune function and muscle metabolism was evaluated in normal and tumor-bearing animals. Male and female Buffalo rats were fed a nutritionally complete, semi-purified diet ± 2% w/w glutamine. Rats trained progressively to swim 4 hours/day or remained sedentary. Tumor-bearing animals received two subcutaneous injections of finely chopped Morris hepatoma 7777 immediately prior to the onset of exercise training.

In normal animals, neither exercise nor L-glutamine supplementation was associated with changes in plasma glutamine concentration, natural killer cytolytic activity, energy metabolism in immune cells or net protein degradation in isolated muscle. The results do not support an increased requirement for glutamine by immune cells during or following exercise in healthy individuals.

In tumor-bearing animals, tumor progression was associated with changes in natural killer cytolytic activity and suppressed cell-mediated immune function. Dietary L-glutamine supplementation, but not exercise, significantly reduced (30%) growth of the Morris hepatoma 7777. Neither exercise nor diet altered splenic natural killer cytolytic activity, but exercise did increase ($p<0.05$) [^{3}H]-thymidine incorporation into splenocytes stimulated with Concanavalin A or Phorbol Myristate Acetate plus Ionomycin. Plasma glutamine concentrations were not affected by diet, exercise or tumor progression. Isolated hepatoma cells demonstrated high rates of non-oxidative glucose and glutamine metabolism and the potential to compete with host tissues for these substrates. The results indicate that (1) the provision of dietary glutamine reduced tumor growth but did not alter plasma glutamine concentrations or energy metabolism in isolated tumor cells and (2) exercise training improved responsiveness to mitogens but did not significantly reduce tumor growth.

This thesis demonstrates that dietary L-glutamine can support host glutamine metabolism and reduce the growth of the Morris hepatoma 7777 in this animal model.

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1. *REVIEW OF THE LITERATURE*

A. *INTRODUCTION*

The metabolic demands of exercise may be associated with adverse effects on immune function. Evidence for these effects has arisen both from epidemiological studies and from laboratory-based investigations of specific immune parameters. Among the consistently observed changes following exercise are leucocytosis, redistribution of circulating lymphocyte populations, an increase in number and activity of natural killer cells and a decrease in plasma immunoglobulin concentrations (reviewed by Ndon et al, 1992). Most of the observed changes appear to be transient since most return to normal within hours after the exercise stimulus. The clinical significance of these changes is not clear, however, it has been suggested that changes in natural immunity due to exercise are beneficial in reducing the risk of neoplasia (MacNeil and Hoffman-Goetz, 1993a; MacNeil and Hoffman-Goetz, 1993b). Clearly, the influence of dietary manipulation on changes in immune function observed during exercise have not been clarified in either the normal individual or the tumor-bearing host.

B. *OVERVIEW OF THE IMMUNE SYSTEM*

1. *Non-specific immunity*: Nonspecific immunity is an organism's initial defence mechanism against an invading pathogen. In addition to various soluble factors (acute phase proteins, complement, lysozymes and interferons), it includes natural killer cells, macrophages, monocytes and polymorphonuclear cells (basophils, eosinophils, neutrophils) (reviewed in Shephard et al, 1991).

Macrophages and monocytes represent different stages of differentiation of the same cell type. In contrast to T cells, macrophages have natural spontaneous cytotoxic reactivity against tumor cell lines (Herberman and Ortaldo, 1981). Among the products of activated macrophages are a variety of enzymes involved in the mechanisms of cytotoxicity, lymphokine activating factors, interferons and prostaglandins (Herberman and Ortaldo, 1981).

Natural killer (NK) cells are functionally identified by their ability to lyse a variety of target cells *in vitro*; morphometrically, they are identified as large granular lymphocytes. Unlike T cells, NK cells do not require the involvement of major histocompatibility antigens to initiate cytotoxicity and there is no evidence of a

memory response (Nieman et al, 1993a). Their activity can be rapidly augmented by interferon and other stimuli and they are thought to be important in the natural resistance against cancer and infectious disease.

2. *Acquired or specific immunity*: The specific immune response amplifies mechanisms of non-specific immunity and enhances their function, particularly upon repeated exposure to the same antigen. The response is initiated by the recognition of a foreign antigen by lymphocytes, which respond by proliferating and differentiating into various types of effector cells.

Both B and T cells recognize and react to antigens. B cells are responsible for antibody production and their characteristics and function have been well defined. T cells are a major subpopulation of lymphocytes that are dependent on the thymus for maturation and acquisition of functional activity (Herberman and Ortaldo, 1981). They have virtually no spontaneous cytolytic activity and must be activated, usually upon exposure to antigens presented on the surface of specialized accessory cells (ie macrophages). There is a considerable latent period before T cells develop their initial reactivity, however they show an accelerated memory response upon re-exposure to an antigen (Herberman and Ortaldo, 1981). Some plant lectins (ie Concanavalin A) can induce polyclonal activation of T cells.

T cells comprise helper (CD4) cells, cytotoxic cells and suppressor (CD8) cells. Helper T cells recognize foreign antigens in the context of the major histocompatibility complex (MHC; Class II) and synthesize lymphokines (interleukin-2, interleukin-3) to further increase T cell activity (Shephard et al, 1991). Cytotoxic T cells recognize and kill cells which have been infected with bacteria or viruses and exhibit foreign antigenic markers on their surfaces (reviewed in Keast et al, 1988). They can lyse tumor cells which have recognizable antigenic changes on their cell surfaces (reviewed in Keast et al, 1988), however, the antigen must be presented to these cells in association with Class I MHC products. Suppressor T cells downregulate the activity of helper and cytotoxic T cells to prevent unnecessary damage to the body.

C. *CHANGES IN LYMPHOCYTE NUMBER AND FUNCTION DURING EXERCISE*

Exercise induces an immediate leucocytosis and marked changes in the composition of blood mononuclear cells (McCarthy and Dale, 1988). It has been proposed that catecholamines produced during exercise increase the ratio of circulating to noncirculating leucocytes (Pedersen, 1991) by reducing endothelial

adhesion of leucocytes to vessel walls (Weicker and Werle, 1991). The spleen, lymph nodes, liver and thymus also carry adrenoreceptors thus their stimulation during exercise might increase leucocyte release from these organs (Weicker and Werle, 1991). From animal experiments, it is known that exogenously administered epinephrine, norepinephrine or isoproterenol releases lymphocytes without altering overall blood flow (reviewed in McCarthy and Dale, 1988). Since changes in lymphocyte number and redistribution are similar to those observed after epinephrine administration, and lymphocytosis can be decreased by β-adrenergic blocking agents, it has been postulated that exercise-induced effects are under β-adrenergic control (Ndon et al, 1992). Adrenergic receptor density differs between lymphocyte subsets and subsets are mobilized selectively during exercise (reviewed in McCarthy and Dale, 1988). Knowledge about the proportional changes in specific subsets is important because it allows understanding of the functional assays in which fixed numbers of mononuclear cells are studied (Tvede et al, 1993).

Glucocorticoids may be responsible for a delayed neutrophilia, causing an increase in the total number of neutrophils in circulation several hours post-exercise (reviewed in McCarthy and Dale, 1988). Glucocorticoids given intravenously cause a reversible redistribution of certain subpopulations from the circulation to other body compartments (Tvede et al, 1989). Changes in leucocyte number during exercise due to plasma volume shifts or hemoconcentration are usually small (McCarthy and Dale, 1988).

Although the total number of lymphocytes increases, exercise may be associated with an overall suppression of immune function. Pahlavani et al (1988) reported a 6 month swimming regime in the rat reduces splenic lymphocyte proliferation to Concanavalin A (Con A) and *in vitro* interleukin-2 production. Similarly, the proliferative response of splenic lymphocytes to Con A was depressed in trained mice relative to sedentary controls (Hoffman-Goetz et al, 1986). In human subjects, both anaerobic (Fry et al, 1992) and submaximal exercise (MacNeil et al, 1991) transiently reduce lymphocyte responsiveness to Con A in peripheral blood, irrespective of subject fitness level. Several mechanisms have been suggested for the post-exercise decrease in mitogen response *in vitro*; soluble factors, including prostaglandins and interleukins, along with hormonal changes and major cell population shifts have been implicated (Fry et al, 1992). Catecholamines produced during exercise increase adenyl cyclase activity and lead to an increase in the intracellular cyclic AMP which

has been found to inhibit both T and B cell responses to mitogens (Keast et al, 1988). Increased glucocorticoid secretion by the adrenal cortex in response to exercise also has suppressive effects on the capacity of mature T-lymphocytes to proliferate to mitogens (Hoffman-Goetz et al, 1989). The time course of recovery may be important for drawing conclusions about whether suppression in lymphocyte cultures following acute exercise is of clinical significance.

Clinical studies have demonstrated an immediate and transient increase in the frequency and activity of natural killer (NK) cells in peripheral blood following acute submaximal or maximal physical work (Simpson and Hoffman-Goetz, 1990; Nieman et al, 1993a; Nieman et al, 1993b). Field et al (1991) reported a transient increase in NK cytolytic activity in human subjects immediately following exhaustive exercise. Simpson and Hoffman-Goetz (1990) reported increased lysis of conventional tumor targets immediately after acute exercise that was depressed two hours later. *In vivo*, the transient suppression of *in vitro* cytotoxicity appears to occur independently of numerical changes in circulating NK cells since normalization of NK cell numbers shortly after the completion of exercise is not accompanied by restoration of cytotoxic function (Pedersen et al, 1988). The underlying physiological mechanisms have not been clarified although, eicosanoids, stress hormones and cytokines have been proposed. The intravenous infusion of epinephrine achieve plasma concentrations similar to those seen during exercise mimicks the exercise-induced effects on NK cell activity (Fitzgerald, 1991). It has previously been shown that prostaglandins released from monocytes are responsible for the post-exercise suppression of NK activity and B cell function, inasmuch as indomethacin *in vitro* abolished the suppression of NK cell activity after exercise (Kappel et al, 1991).

Exercise training may activate NK cells for an extended time or may increase the sensitivity of NK cell receptors to immunopotentiating hormones and lymphokines (Simpson and Hoffman-Goetz, 1990). The clinical significance of these observations is not clear, but it is conceivable that frequent enhancement of cytotoxic mechanisms during and after acute exercise could be beneficial in preventing infection or neoplasia. In experimental animals, exercise has been shown to enhance resistance to tumor growth (Baracos 1989). It is possible that athletes may be more resistant to cancer by a similar mechanism. Further research is warranted to establish if factors such as interferon or interleukin-2, which are potent stimulators of NK cells, play a role.

D. PHYSIOLOGICAL IMPORTANCE OF GLUTAMINE

A requisite demand for glutamine exists in a variety of tissues: it is an energy substrate for the gastrointestinal tract, a precursor for purine and pyrimidine nucleotides in rapidly dividing cells and an important interorgan carrier of ammonia (reviewed in Bulus et al, 1989). Stimulated cells of the immune system have high energy needs and accelerated rates of protein and nucleic acid biosynthesis (reviewed in Lacey and Wilmore, 1990). When lymphocytes are activated by an immune stimulus, they are transferred into a high state of biochemical activity (Ardawi and Newsholme, 1984). This involves both rapid cell division and the production of many soluble mediators of cellular immunity (Ardawi and Newsholme, 1984). The importance of glutamine metabolism in lymphocytes is indicated from the observation that these cells possess high activities of glutaminase and that this activity is increased *in vivo* during an immunological challenge (Ardawi and Newsholme, 1983; Caldwell 1989). *In vitro*, glutaminase activity in lymphocytes isolated from rat mesenteric lymph nodes increased following mitogenic stimulation by Concanavalin A (Ardawi and Newsholme, 1983).

In activated lymphocytes, glutamine mostly undergoes partial oxidation to lactate, ammonia, glutamate and aspartate (Ardawi and Newsholme, 1983; Brand et al, 1989). This process involves the flux of glutamine carbon chain through the second half of the citric acid cycle (from α-ketoglutarate to pyruvate via malate) and the generation of adenosine triphosphate (ATP). Besides providing energy, this process provides ammonia and aspartate, required for purine and pyrimidine synthesis, and nitrogen for the formation of glucosamine, guanosine triphosphate (GTP) and nicotinamide adenine dinucleotide (NAD) (reviewed in Lacey and Wilmore, 1990). Ardawi and Newsholme (1985) studied lymphocytes from rat mesenteric lymph nodes and established the relationship between glutamine utilization and cell proliferation. Adding glutamine to the culture resulted in a four-fold increase in[^{3}H]-thymidine incorporation that could not be duplicated by replacing glutamine with any other amino acid or by ammonia. Reasons for the importance of glutamine in tissue culture include the roles of glutamine in essential transamination reactions, as an intermediate in nucleic acid synthesis and as a component of cellular protein (Lacey and Wilmore, 1990).

It is unclear whether glutamine metabolism is more important for the provision of energy or the provision of carbon and nitrogen precursors for biosynthetic processes

in lymphocytes (Ardawi and Newsholme, 1985). Since the rate of glutamine oxidation in rapidly dividing lymphocytes is higher than the rate of utilization for biosynthesis of macromolecules, it has been suggested that the major role of glutamine utilization under this condition is energy provision (Ardawi and Newsholme, 1985). The rate of glutamine utilization at the physiological blood concentration (0.5 mM) is approximately 55 μmoles/hour per 10^{10} cells (Ardawi and Newsholme, 1984). For the total *in vivo* population of lymphocytes of a 200 g rat (approximately 4 x 10^9 lymphocytes including those found in lymph nodes, spleen, thymus, bone marrow and peripheral circulation) the rate of utilization for the biosynthesis of purines and pyrimidines is about 4% of the *in vitro* rate of glutamine utilization by incubated lymphocytes (Ardawi and Newsholme, 1984).

Glutamine is made available in the lumen of the intestine from the digestion of protein, however, enterocytes utilize glutamine at a high rate and could potentially utilize all that is absorbed (Ardawi and Newsholme, 1984). Evidence suggests that glutamine derived from blood is an important respiratory substrate for cells in the mucosa of the small intestine and that the intestine is a major site for the metabolism of glutamine released into the circulation by other tissues (Windmueller and Spaeth, 1974). Glutamine is metabolized similarly whether it enters the mucosal cells across the brush border which interfaces with the lumen or across the basolateral membrane from arterial blood (Salloum et al, 1991). Large arteriovenous differences were found for glutamine across the intestine in rat, dog, cat, hamster and monkey and the fall in glutamine concentration observed across the rat intestine *in vivo* was similar to that observed across the isolated preparations (Windmueller and Spaeth, 1974). Arteriovenous measurements like those for the intestine made in fasted rats across most large organs or body regions including liver, kidney, lungs, heart, head and hindquarters failed to reveal any organ, other than the intestine, in which an appreciable net extraction of glutamine occurred (Windmueller and Spaeth, 1974). The main source of circulating glutamine in fasting animals appears to be skeletal muscle. In rats, the hindquarter released 48 μmoles of glutamine per hour or 64% of the net uptake by the intestine (Windmueller and Spaeth, 1974). Glutamine uptake comparable to that found in the isolated organ was observed *in vivo*, from arteriovenous concentration measurements across the intestine in fasted animals. Twenty-five to 33% of plasma glutamine was extracted in each pass through the intestine (Windmueller and Spaeth, 1974). The above evidence suggests a high

cellular priority and requirement for glutamine by the small intestine exists, hence oral or intravenous glutamine supplementation may not reach skeletal muscle in concentrations sufficient to increase intramuscular glutamine concentrations (Wu and Thompson, 1990). The efficacy of dietary glutamine provision has not been established in this regard in normal animals thus glutamine production and release by muscle becomes of considerable physiological and immunological importance.

E. GLUTAMINE RELEASE FROM SKELETAL MUSCLE

Most of the glutamine present in blood is synthesized and released by skeletal muscle; it contains high concentrations of glutamine, it has the enzymatic capacity to synthesize glutamine and it is known to release glutamine into the blood stream at high rates (reviewed in Parry-Billings et al, 1990). Therefore, it is likely that all or most of the glutamine utilized by lymphocytes originates in muscle (Ardawi and Newsholme, 1984). Generally, the magnitude of glutamine release by muscle exceeds that which is required by lymphocytes. The concentration of glutamine required to stimulate proliferation by 50% of the maximal rate is about 0.02 mM in both rat and human lymphocytes (Parry-Billings et al, 1990). A tenfold increase in substrate above Km provides 91% of the maximal rate. Hence, at the normal plasma concentration of glutamine (0.5 mM in man and 0.9 mM in the rat, the rate of proliferation should be close to maximal (Parry-Billings et al, 1990). Based on *in vitro* work, one might predict that decreases in plasma glutamine concentration below 0.2 mM might impair the rate of lymphocyte proliferation. Hence, if the rate of glutamine release by muscle is decreased during a particular pathological (ie tumor competition) or physiological condition (ie exercise), plasma glutamine concentrations may decrease and the lymphocytes could potentially be forced into glutamine debt. This could result in an impaired ability of lymphocytes to respond to an immune challenge by increasing the rates of purine and pyrimidine nucleotide synthesis for DNA and RNA formation (Parry-Billings et al, 1990). The problem may be more severe than indicated since the concentration of glutamine in body fluids (ie saliva, lymph, peritoneal fluid) may be lower than in plasma (Parry-Billings et al, 1990).

Recent studies on muscle glutamine levels and its release suggest that the key process in controlling the rate of glutamine release by muscle is a glutamine transporter that specifically transports glutamine across the cell membrane (Parry-Billings et al, 1990). The concentration of glutamine in skeletal muscle is thirty times

greater than the concentration in blood, therefore the gradient favors the export of glutamine (reviewed in Lacey and Wilmore, 1990). Any decrease in the muscle glutamine concentration could reflect decreased activity of glutamine synthetase, increased glutaminase activity or changes in the number of activity of glutamine transporters (Millward et al, 1988). Because of the sensitivity of the transporter, considerable changes in muscle glutamine concentrations can occur in response to a variety of insults, including exercise (Millward et al, 1988). The muscle transporter appears to be specific for glutamine, histidine and asparagine and is sodium-dependent with a coupling ratio of 1Na:1Gln (Rennie et al, 1989). Because the maintenance of the glutamine gradient is sodium dependent, factors that influence sodium transport, either by changing the activity of Na+K+ATPase or by influencing membrane permeability to Na+ could influence the glutamine gradient (Millward et al, 1988). In general, the rate of transport can be influenced by hormones, metabolites and changes in electrolyte gradients occurring in various physiological and pathological conditions (Parry-Billings et al 1990).

The release of glutamine by skeletal muscle is inhibited by catecholamines and is mediated by β-adrenergic stimulation of skeletal muscle adenyl cyclase activity and increased cAMP levels (Garber, 1980). Garber et al (1976) demonstrated that the addition of epinephrine to muscle preparations from fed rats markedly decreased the rate of release of glutamine and that the magnitude of inhibition was proportional to the log dose of epinephrine. The lowest epinephrine concentration producing measurable inhibition of release was 10^{-9} M and the degree of inhibition continued to increase with epinephrine concentrations up to 10^{-5} M. Activation of muscle glycogenolysis, as indicated by increased rates of lactate release, was not observed with concentrations of epinephrine less than 10^{-8} M; this is significant since this is the lowest concentration at which the physiological effects of exercise could be reproduced *in vitro*. Norepinephrine inhibited amino acid release in a similar manner to epinephrine, but at least a 100-fold greater concentration was required to produce the same degree of inhibition obtained with epinephrine. The large differences in biological activity between epinephrine and norepinephrine suggest that catecholamine inhibition of amino acid release represents a β-adrenergic effect (Garber et al, 1976). This hypothesis was tested using α- and β-adrenergic agonist and antagonists. The β-adrenergic agonist, in equimolar concentrations, reproduced the epinephrine inhibition of glutamine release, however, the α-adrenergic agonist

had no observable effect on glutamine release from skeletal muscle (Garber et al, 1976; Garber et al, 1984). Epinephrine (10^{-5} M) also increased tissue levels of cyclic AMP in muscle preparations and these increases were associated with marked inhibition of glutamine release such that the intermediary participation of muscle adenylate cyclase and intracellular cyclic AMP became apparent. Increased tissue levels of cyclic AMP were not observed with lower concentrations of epinephrine which inhibited glutamine release to a lesser extent (Garber et al, 1976). The physiological importance of adrenergic inhibition of glutamine synthesis is dependent upon the demonstration that the catecholamine levels required for inhibition *in vitro* are consistent with hormone levels *in vivo*. In a study by Pedersen (1991), healthy volunteers were either exercised on a bicycle ergometer (75-80% VO2max for one hour) or given epinephrine via intravenous infusion to obtain plasma levels comparable to those seen during exercise. The results showed that modulation of lymphocyte function during exercise was closely mimicked by the administration of exogenous epinephrine (Pedersen, 1991). Comparing the *in vivo* levels to the concentration dependent action of epinephrine on muscle amino acid release observed *in vitro*, it is apparent that physiological changes in the circulating levels of epinephrine *in vivo* could be associated with the altered rates of glutamine release from skeletal muscle (Garber et al, 1976) and with alterations in lymphocyte function.

The literature suggests that the mechanism of epinephrine action does not involve the inhibition of amino acid efflux, however. Tissue levels of glutamine would be expected to increase as consequence of decreased transport across the cell membrane (Garber et al, 1976). The significant decrease in tissue amino acid levels that is actually observed implies that the amino acid release is decreased as a result of a reduction in the rate of net amino acid synthesis (Garber et al, 1976). Karl et al (1976) studied glutamine formation and release from intact epitrochlearis preparations of rat skeletal muscle. The addition of glutamine to the incubation medium did not significantly alter the tissue levels of glutamine during incubation. Since the intracellular pools of glutamine did not change during incubation, the release rate of glutamine under the conditions of study must have reflected their rate of synthesis (Karl et al, 1976). Three potential mechanisms by which epinephrine could inhibit glutamine synthesis in skeletal muscle include (a) a reduction in the rate of amino acid transport out of the muscle cell (b) an inhibition of precursor amino acid conversion to glutamine (c) reduced availability of precursor amino acids secondary

to decreased degradation of endogenous muscle proteins (Garber et al, 1976).

F. EXERCISE EFFECTS ON PLASMA GLUTAMINE

In human subjects, the response of plasma glutamine to exercise varies according to the duration of exercise. Rennie et al (1981) demonstrated that plasma glutamine levels were elevated at the onset of exercise but fell progressively as the exercise continued and remained slightly depressed throughout the recovery period. In this study, subjects exercised on a treadmill at 50-60% VO2max for approximately four hours. To some extent, similar results have been observed in rats; plasma glutamine levels decreased after exercise to exhaustion in trained rats but were unchanged following exercise in sedentary rats which ran for a significantly shorter time (Parry-Billings et al, 1990). When the soleus muscle was isolated from exhausted rats and incubated *in vitro*, the rate of glutamine release was less than that from muscles removed from resting animals (Parry-Billings et al, 1990). The decrease in plasma glutamine concentration following long-term exercise is consistent with other studies of prolonged exercise in both human subjects and experimental animals.

The changes observed following an exhaustive bout of exercise, which last for several hours, are seen in both athletes and untrained individuals. Similar effects presumably occur each time an athlete exercises. If exercise periods are too frequent, too intense and/or too prolonged, recovery after each exercise bout will be incomplete and could result in an additive impairment of the immune system. Parry-Billings et al (1990) reported significantly lower plasma glutamine concentrations in overtrained athletes. A subset of overtrained subjects and their matched controls were tested after a six week recovery period and despite an improvement in exercise performance, plasma glutamine concentrations remained below control values in the overtrained subjects and were not affected by the recovery period (Parry-Billings et al, 1990). The data suggests that the effects of overtraining persist for longer periods of time than the decrease in physical performance and that overtraining might chronically alter plasma glutamine concentrations and immune function.

An *in vivo* depletion of glutamine in rats has been shown to be immunosuppressive, however, the immunosuppression can be reversed by normalizing plasma glutamine concentrations or by the addition of glutamine to *in vitro* cultured lymphocytes (Ardawi, 1988; Kafkewitz and Bendich, 1983). *In vitro*, lowered concentrations of glutamine adversely affect lymphocyte proliferation and

macrophage function, even in the presence of alternate fuels (Sharp, 1992). The rate of proliferation *in vitro*, assessed by ^{3}H-thymidine incorporation into Concanavalin A stimulated peripheral lymphocytes over 48 hours at a range of glutamine concentrations (1.0, 0.6, 0.3 mM) was not significantly different between overtrained subjects and matched controls suggesting the contribution to immunodeficiency was from decreases in plasma glutamine affecting lymphoid and macrophage function rather than from the intrinsic function of the cells being impaired (Sharp, 1992). Kafkewitz and Bendich (1983) uniquely depleted glutamine using bacterial enzymes to demonstrate that glutamine depletion alters immune function.

If inadequate plasma glutamine concentrations are shown to be immunosuppressive, athletes training intensively may need to take dietary supplements. Dietary glutamine may be administered in concentrations sufficient to increase intramuscular concentrations, however the required dose will depend on the extraction of glutamine by cells of the intestinal mucosal. Depressed glutamine concentrations may have lesser effects on immune function if lymphocytes are able to derive energy from other circulating metabolites in blood during exercise.

Literature relating plasma glutamine levels to exercise and immune function is limited. This is possibly because of the inherent difficulty in conducting a study involving glutamine. Glutamine, unlike most other amino acids, is heat-labile, relatively unstable in aqueous solution and sensitive to an acid environment (Lacey and Wilmore, 1990). In solution, it undergoes ring condensation to form pyroglutamic acid and ammonia and in the presence of heat or strong acid or base, glutamine breaks down to glutamic acid and ammonia (Lacey and Wilmore, 1990). Such degradation occurs readily during the routine processing of plasma, whole blood or other biological fluids prepared for amino acid analysis. In addition, the current literature investigating hormone regulation during exercise has focused mainly on hormonal-metabolic communication and less on hormonal-immunologic communication. Finally, conclusions about the effects of exercise on immune function are controversial. This is likely caused by differences in the intensity and duration of exercise protocols and by the associated hormonal and metabolic changes, by the training level of the subjects and by the methods employed to measure immune function. The controversial results and the contravening variables make it difficult to accurately predict whether dietary glutamine supplementation would be effective in enhancing the immune response to exercise. Moreover, in the

normal individual, there is no data supporting an increased requirement for glutamine by lymphocytes during or following exercise.

G. *METABOLIC ALTERATIONS IN THE TUMOR-BEARING HOST*

The presence of a tumor alters the metabolic and hormonal environment of the host by successfully competing with normal tissues for important metabolites, energetic substrates and trophic factors. It also influences some host tissues by decreasing differentiation, changing enzyme characteristics and sensitivity to hormones, and by disturbing negative feedback systems (Argiles and Lopez-Soriano, 1991). With progressive tumor growth, altered glutamine metabolism becomes a characteristic feature of the tumor-bearing host (Souba et al, 1988). The accelerated muscle catabolism and loss of lean body tissue commonly associated with cancer cachexia may reflect the tumor's ability to alter amino acid metabolism in the liver and intestine and to redirect the flow of amino acids away from skeletal muscle (Souba et al, 1988). Skeletal muscle glutamine concentrations correlate directly with skeletal muscle protein synthesis and indirectly with muscle protein degradation (Austgen et al, 1992; Medina et al, 1992). Parry-Billings et al (1990) observed a rapid and marked decrease in skeletal muscle glutamine content which correlated with the size of the tumor and a decrease in plasma glutamine concentrations. Chen et al (1993) demonstrated that tumor growth stimulates a two-fold increase in hindquarter glutamine release and a simultaneous increase in the activity of glutamine synthetase. It is hypothesized that increased rates of muscle glutamine release during cachexia represent a response of this tissue to satisfy the demand for glutamine or glucose by the tumor (Parry-Billings et al, 1990). Alternately, the increased rate of glutamine release from muscle in response to tumor-bearing could be to provide glutamine for cells of the immune system which may be stimulated in response to tumor invasion.

Besides leading to muscle glutamine depletion, the tumor may compete with the intestinal tract for circulating glutamine (Klimberg et al, 1990). The ability of the tumor to modulate intestinal glutamine metabolism is important because the small intestine is the principle organ of glutamine consumption. Intestinal extraction of circulating glutamine across the basolateral membrane is diminished in the tumor-bearing rat, however selective stimulation of brush border glutamine transport persists even when the tumor is very large and cachexia is evident (Salloum et al, 1991). This upregulation appears specific for glutamine since transport of other amino acids and

glucose is either unaltered or diminished (Salloum et al, 1991).

The liver is the principle organ of amino acid metabolism and plays a key role in maintaining glutamine homeostasis (Chen et al, 1993). In addition to utilizing circulating glutamine, the growing tumor can modulate enzymatic regulation of hepatic glutamine metabolism and thus impair glutamine homeostasis (Chen et al, 1993). Previous studies have demonstrated that the liver switches from an organ of glutamine balance to one of glutamine release with progressive malignant growth (Chen et al, 1993). Circulating glucose concentrations are unaffected by the presence of the tumor, owing to either liver glycogenolysis or enhanced gluconeogenesis (Argiles and Lopez-Soriano, 1991). Classic renal adaptations to mild systemic acidemia are not present in tumor-bearing rats (Chen et al, 1993). Rather, renal glutamine synthetase activity is enhanced despite reduced net renal glutamine uptake (Chen et al, 1993).

Plasma amino acid profiles in neoplasia are influenced by amino acid uptake or release by the tumor and by the catabolism of normal body tissues (Chance et al, 1988). Amino acid balance can be disrupted in the presence of a tumor by: variations in ingested protein, changes in intestinal absorption, alterations in non-essential amino acid biosynthesis in the liver, changes in tissue oxidative breakdown of amino acids and tumor demand for amino acids needed for growth (Marquez et al, 1989). Moyer and Pitot (1974) found several amino acids, especially glutamine, threonine, alanine and lysine, to be decreased in the plasma of hepatoma bearing rats. It is however difficult to attribute an observed change in plasma glutamine concentration to one or any of the above metabolic processes without more detailed organ balance studies.

H. SUBSTRATE UTILIZATION IN NEOPLASTIC TISSUES

Tumors demonstrate an ability to utilize whatever substrate is in most abundant supply (Sauer et al, 1982). Glucose, glutamine and lactate utilization correlate either with supply or the arterial blood concentrations (Sauer et al, 1982). Glutamine is both an important respiratory fuel and a requisite substrate for de novo nucleotide biosynthesis in neoplastic tissues (Austgen et al 1992, Kallinowski et al, 1987). Pi-glutaminase is localized in the inner mitochondrial membrane and catalyzes the hydrolysis of glutamine (Medina et al, 1992). Koracevic and Morris (1972) studied energy metabolism in a series of hepatomas of different growth rates and degrees

of differentiation; a correlation between the activity of mitochondrial glutaminase and the rate of respiration of tumor mitochondria in the presence of glutamine was observed. They also found that the Km of glutaminase in intact mitochondria isolated from a series of Morris hepatomas was inversely correlated with the rate of tumor growth. This is agreement with the findings of Knox et al (1979) who demonstrated a close correlation between glutaminase activity and the degree of histological differentiation in certain tumors. In rapidly growing hepatomas, low intracellular glutamine concentrations can be partially attributed to the increased activity of glutamine utilizing enzymes of purine and pyrimidine biosynthesis, to the high activity of glutaminase and to the low activity of glutamine synthetase (Medina et al, 1992).

An adequate supply of glutamine appears to be necessary for tumor growth and blockade of glutamine utilization leads to a dramatic decrease in the growth rate of experimental tumors, both *in vitro* and *in vivo* (Chance 1988; Chance et al, 1990). The reduction in growth of transplantable rat hepatomas observed following treatment with the anti-glutamine metabolite acivicin appeared to be directly related to inhibition of enzymes necessary for the biosynthesis of purine and pyrimidine nucleotides from glutamine. The activities of these enzymes are reportedly increased in rat hepatomas, thus their inhibition may be considered desirable targets for cancer chemotherapy (Chance, 1990).

Glutamine is also an alternate substrate to glucose for metabolism to pyruvate (Tannock et al, 1986). There is, however, a lack of information about the relative importance of glutamine and glucose for energy provision in tumor cells. As in lymphocytes, energy needs alone do not fully explain the high rates of glycolysis and glutaminolysis because the oxidation of both substrates is only partial (Medina et al, 1988). High rates of glutaminolysis and glycolysis may provide optimal conditions for the precise regulation of the biosynthetic processes that use intermediates of the pathways (Board and Newsholme, 1990). Enzyme capacity is not responsible for low rates of oxidation by the citric acid cycle, hence knowledge about the metabolic control of these enzymes may be of considerable importance in understanding energy formation in these cells (Board and Newsholme, 1990). Tumors metabolize glucose via glycolysis at a high rate even in the presence of normal oxygen availability (Argiles and Azcon-Bieto, 1988). Supplying tumor cells with glucose results in an inhibition of oxygen consumption which magnifies the dependence on glucose conversion to lactate for energy (Argiles and Azcon-Bieto, 1988). As a result of high

glycolytic rates, large amounts of pyruvate and an increase in the cytosolic NADH/NAD+ ratio is generated. Steady-state lactate and pyruvate concentrations are actively maintained in the cytosol by lactate dehydrogenase; intracellular concentrations equilibrate with arterial blood and the steady state is expressed in venous blood (Argiles and Azcon-Bieto, 1988). Lactate utilization by the tumor occurs when arterial concentrations exceed intracellular concentrations (Sauer and Dauchy, 1985).

Ketone bodies can also be important respiratory fuels in undifferentiated tumors. Arteriovenous differences for acetoacetate, β-hyroxybutyrate, glucose, lactate, glutamine and other amino acids were measured across Morris hepatoma 7777's *in vivo* in rats fasted for 48 hours (Sauer and Dauchy, 1983). The rate of ketone body utilization was dependent on the rate of supply (Sauer and Dauchy, 1983). Decreased total arterial whole-blood ketone body concentrations suggested that the presence of the tumor decreased the ketogenic response of the tumor-bearing host to starvation (Sauer and Dauchy, 1983).

I. NUTRITIONAL SUPPORT OF THE TUMOR-BEARING HOST

Nutritional support of tumor-bearing organisms has been studied in both laboratory research and clinical practice. It has been hypothesized that nutrient administration in the host will selectively feed the tumor and potentiate its growth while progressive anorexia and cachexia limit the host's ability to mobilize its natural anti-tumor defence mechanisms (Istfan et al, 1992). It has also been hypothesized that nutritional support in the cancer patient may improve anti-tumor host defence mechanisms and reduce tumor growth (Istfan et al, 1992). To support the first hypothesis, numerous animal studies clearly demonstrate that both oral and parenteral nutrition can significantly stimulate tumor growth and metastasis (reviewed in Torosian, 1992). Tumor tissues have a high affinity for the uptake of amino acids and glucose and therefore the tumor consumes a large portion of the supplied nutrients (Chance et al, 1990). The stimulation of tumor growth by TPN is not obligatory and depends on its metabolic components and constituents (Fischer and Chance, 1990).

Protein-calorie deprivation in animals causes a reduction in spontaneous tumorigenesis and decreased establishment of transplanted tumors (reviewed in Torosian, 1992). Energy restriction has been shown to independently inhibit the growth of spontaneous, transplanted and chemically induced tumors in rats and mice

(Kritchevsky, 1990). It is evident that 40% caloric restriction inhibits tumorigenesis even when restricted rats ingest twice as much fat as *ad libitum* fed controls (Kritchevsky, 1990). It is also possible that manipulation of non-essential amino acids under conditions of limited total substrate availability could also independently influence tumor and host carcass weight (Popp et al, 1988). The same degree of tumor depression produced by nitrogen deprivation can also be produced by removal of asparagine, glutamine and their precursors, glutamate and aspartate from TPN solutions without adverse effects on carcass mass (Popp et al, 1988). This represents a process other than simple lowering of total nitrogen by which selective starvation of the tumor may be obtained with minimum effects on the host carcass (Popp et al, 1988).

Attempts to use dietary manipulation to selectively support the patient or combat the tumor have an underlying set of hypotheses: a tumor-bearing host has different metabolic and nutritional demands than a non-tumor-bearing host and that there are differences in the metabolism and nutrient requirements between a malignant cell and a normal cell of the same origin (Argiles and Azcon-Bieto, 1988). A better understanding of host and tumor competition for limited quantities of substrate might allow the design of innovative new forms of cancer therapy. Theoretically, any nutritional therapy that would attenuate the net muscle proteolysis which is characteristic of advanced malignant disease would be beneficial if tumor growth is not stimulated and tolerance to anti-neoplastic therapy is enhanced (Klimberg et al, 1990).

J. THERAPEUTIC BENEFITS OF GLUTAMINE IN THE TUMOR-BEARING HOST

In support of the second hypothesis of nutritional support, Klimberg et al (1990) examined the effects of oral glutamine on muscle glutamine metabolism and tumor growth in rats. The significant increase in muscle glutamine concentration and hindquarter release in glutamine-fed animals was explained by a stimulation of *de novo* intracellular glutamine synthesis and/or augmented uptake of glutamine by skeletal muscle occurring during the post-prandial state. Blood concentrations were not altered by dietary glutamine supplementation. Moreover, tumor glutaminase activity was not altered by the glutamine-enriched diet (Klimberg et al, 1990).

To evaluate the effects of a glutamine-supplemented diet on skeletal muscle synthetase activity and tissue glutamine concentrations, a group of tumor-bearing rats

had their drinking water supplemented with glutamine (Chen et al. 1993). A second group of rats with similar size tumors were given an isonitrogenous drinking solution containing a mixture of three other non-essential amino acids (serine, alanine and glycine). Hindquarter blood flow, glutamine flux, skeletal muscle glutamine concentrations and glutamine synthetase activity between control and tumor-bearing rats were not different in animals when the tumor burden was small. In animals with a large tumor burden, arterial glutamine concentration was significantly depressed in tumor-bearing rats compared to controls and glutamine feeding increased muscle glutamine concentrations and glutamine synthetase activity.

In another study, provision of 20% of TPN protein as glutamine produced a significant increase in arterial glutamine levels and maintained skeletal muscle intracellular glutamine concentrations (Austgen et al. 1992). Glutamine-enriched TPN did not affect carcass weight, tumor weight, tumor DNA content or tumor glutaminase activity but did increase gut mucosal glutathione (GSH) levels (Austgen et al. 1992). Glutamine-enriched TPN may therefore be beneficial to host tissues by repleting intracellular muscle glutamine concentrations, by maintaining muscle glutamine efflux and by supporting gut glutathione levels. Overall, these studies support the suggestion that glutamine may have therapeutic as well as nutritional advantages in cancer patients.

The administration of glutamine-enriched TPN benefits healthy animals by supporting gut mucosal metabolism, structure and barrier function (Klimberg et al. 1990). Given the intimate relationship between skeletal muscle and intestinal glutamine metabolism, additional amounts of glutamine from exogenous or endogenous sources may be required to maintain the mucosa of the small intestine during periods of glutamine depletion (Souba and Austgen, 1990). Fox et al (1987) described the benefits of a glutamine-enriched diet in rats with methotrexate-induced entercolitis. The experiment showed that a glutamine-enriched elemental diet significantly improved nutritional status, decreased intestinal injury, decreased bacterial translocation and minimized mortality. Glutamine can also enhance the tumoricidal effectiveness of methotrexate (Klimberg et al. 1992a; Klimberg et al. 1992b). Significant differences in tumor glutaminase activity and morphometrics were not detectable with glutamine supplementation in methotrexate-treated rats and the effects could not be attributed to differences in energy or nitrogen intake since all diets were isocaloric and isonitrogenous. Additional studies designed to examine the

effects of glutamine-enriched nutrition on mucosal growth and function in the rat with advanced malignant disease are required.

K. EXERCISE AND EXPERIMENTAL TUMOR DEVELOPMENT

Exercise can alter the course of malignancy in experimental animals, however the impact of exercise may strongly be influenced by the tumor model (carcinogen, implant, spontaneous), the phase of neoplasia (initiation, promotion, progression) and the timing of the exercise (before, during, after onset of neoplastic lesion) (reviewed in MacNeil and Hoffman-Goetz, 1993b). Most reports on exercise and experimental tumors have either continued or begun exercise training during the tumor growth period and have typically focussed on initiation and promotion of chemically-induced tumors (reviewed in MacNeil and Hoffman-Goetz, 1993b). Clear evidence linking physical activity with cancer resistance comes from studies of DMBA or NMU-induced mammary tumors. Voluntary exercise administered during the promotion phase of carcinogenesis inhibits the development of mammary tumors whereas forced exercise promotes tumorigenesis (MacNeil and Hoffman-Goetz, 1993a). Cohen et al (1988), using a voluntary running protocol, showed decreased tumor yield and delayed time of tumor appearance in rats treated with N-nitrosomethylurea (NMU). With respect to transplanted tumors, exercise inhibits (Baracos, 1989) or has little impact on primary tumor growth (MacNeil and Hoffman-Goetz, 1993b). A significant reduction in the size and protein content of rat hepatomas three weeks after implantation is observed whether the tumor is implanted into an exercising animal or into a sedentary animal prior to the onset of exercise (Baracos, 1989). Chronic exercise discontinued and followed by intravenous injection of tumor cells does not reduce the subsequent incidence of tumor metastases in mice (MacNeil and Hoffman-Goetz, 1993b). Tumor volume, tumor weight, cellular mitotic index, DNA, RNA and protein synthesis and cell cycle changes have been used as parameters of tumor growth. Different techniques to describe tumor growth characteristics as well as the complexity of quantifying tumor growth characteristics *in vivo* have significantly contributed to this controversy (Istfan et al, 1992). Moreover, many studies with rodents fail to quantify the metabolic cost of work or the physiological effects of the exercise programs making comparability of the results difficult. There is a definite need to define the metabolic consequences of exercise in order to clarify and quantify true effects of exercise on immune function.

Various mechanisms have been proposed to explain the effects of exercise on cancer development: enhanced antioxidant function, changes in hormonal or immunological status, altered prostaglandin metabolism and reduced nutrient availability (Baracos, 1989). The rate of metastasis is inversely related to the level of natural killer cell function in mice (MacNeil and Hoffman-Goetz, 1993b). It has been hypothesized that changes in natural immunity due to exercise are clinically beneficial in reducing the risk of neoplasia (MacNeil and Hoffman-Goetz, 1993b), however systematic evidence about causal relationships is absent from the literature. It is also known that infection in mice causes a significant elevation of serum TNF which is attenuated by a daily swimming program (Chao et al, 1992). The effects of this exercise protocol on other cytokines (interferon-gamma and interleukin-1) are not known. Additional research is required to define the mechanisms by which exercise inhibits or promotes tumor growth.

L. *CONCLUSIONS*

In the normal individual, there is no evidence supporting an increased requirement for glutamine by immune cells during or following exercise nor is there information that dietary supplementation can alter glutamine concentrations in plasma or muscle. In the tumor-bearing individual, additional studies designed to investigate the consequences of glutamine-enriched nutrition are required, particularly as they relate to exercise-induced alterations in immune function.

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2. *RESEARCH PLAN*

A. RATIONALE

Exercise stress induces marked changes in the composition and function of blood mononuclear cells. As most studies have examined changes in blood, little is known about how exercise affects the activity of immune cells in lymphoid organs, such as spleen.

Stimulated cells of the immune system have high energy requirements and accelerated rates of protein and nucleic acid biosynthesis. Considerable evidence exists that glutamine is a requisite energy substrate and a precursor of purine and pyrimidine nucleotides in these cells. The requirement for glutamine by immune cells during or following exercise has not been established.

It has been suggested that a decreased efflux of glutamine from skeletal muscle during intense exercise of long duration results in decreased plasma glutamine concentrations. The possible effect of these decreases on immunocompetence has been proposed as a mechanism for the increased incidence of infection among athletes. Despite an absence of information demonstrating that small fluctuations in plasma glutamine concentration alter immunocyte function and that glutamine supplementation is effective in increasing plasma and/or muscle concentrations in healthy individuals, it has been recommended that athletes receive glutamine supplementation.

An adequate supply of glutamine also appears to be necessary for tumor proliferation since blockade of glutamine utilization leads to a dramatic decrease in the growth rate of experimental tumors. Clearly, the clinical significance of transient changes in immune status and glutamine homeostasis during exercise has not been established, particularly as it affects resistance to neoplasia. Such information will be important in designing therapeutic intervention programs in some cancer patients.

B. OBJECTIVES

The objectives of this thesis are:

1. to characterize the effects of exercise on lymphocyte metabolism and function;
2. to establish the requirement for glutamine by immune cells during or following exercise;

3. to determine if dietary L-glutamine supplementation alters changes that occur in muscle protein metabolism and immune function during exercise;
4. to evaluate the temporal relationship between tumor development and immune responsiveness;
5. to examine concurrently the effects of dietary L-glutamine supplementation and exercise on specific parameters of immune function and experimental tumor development;
6. to assess the relative importance of glucose and glutamine as energy substrates in freshly isolated Morris hepatoma 7777 cells;

C. *HYPOTHESES*

It was hypothesized that:

1. in the non-tumor-bearing animal, diet and/or exercise would alter immune cell and muscle metabolism;
2. in the tumor-bearing animal, the provision of dietary L-glutamine would support skeletal muscle metabolism and immune function during exercise without stimulating tumor growth;
3. in the tumor-bearing animal, exercise would alter the course of tumor development via changes in natural immune function, immune responsiveness and/or tumor cell metabolism;

D. *CHAPTER FORMAT*

The hypotheses posed were tested in a sequence of experiments. The experiments have been organized as thesis chapters and will be submitted for scientific publication as individual papers. A general discussion with potential other areas of research is presented in Chapter 6.

3. *DIETARY L-GLUTAMINE SUPPLEMENTATION AND EXERCISE DO NOT ALTER IMMUNE FUNCTION OR NET PROTEIN DEGRADATION IN MUSCLE*

A. INTRODUCTION

Glutamine occupies a central role in a variety of metabolic processes. It is a major substrate in pathways of renal ammoniagenesis and a precursor of purine and pyrimidine nucleotides (reviewed in Smith, 1990). It is a potential regulator of glycogen synthesis in the liver (Katz et al, 1976) and of protein turnover in skeletal muscle (MacLennan et al, 1987). Moreover, it is an important metabolic substrate for cells of the intestinal mucosa (Windmueller, 1982) and for rapidly proliferating cells (Ardawi and Newsholme, 1984). The importance of glutamine in lymphocytes is indicated from the observation that these cells possess high glutaminase activity which increases *in vivo* in response to an immunological challenge and *in vitro* in response to mitogenic stimulation (Ardawi and Newsholme, 1983).

Following surgery, trauma, sepsis or in other highly catabolic states, plasma and tissue intracellular glutamine concentrations are rapidly and severely depleted (reviewed in Smith and Wilmore, 1990). Under such circumstances, a glutamine-enriched elemental diet significantly improves nutritional status, induces trophic effects on the lumen of the small intestine and reduces mortality (Fox et al, 1987). Because a large proportion of glutamine in blood is derived from skeletal muscle (Smith, 1990) and skeletal muscle glutamine release may decrease during intense exercise of long duration (Parry-Billings et al, 1990), the efficacy of glutamine-supplementation in maintaining host immunocompetence during exercise has been questioned. The requirement for glutamine by cells of the immune system during exercise is not known nor has it been established that small fluctuations in plasma glutamine concentration impair lymphocyte function. The objective of the current study was to characterize the effects of exercise on lymphocyte metabolism and function and to determine whether dietary L-glutamine supplementation would alter changes in muscle protein metabolism and immune function during exercise.

B. MATERIALS AND METHODS

Animal protocol [experiment one]:

Studies were carried out in accordance with the guidelines of the Canadian Council on Animal Care. Thirty-six male and female Buffalo rats were randomly

assigned to two groups and fed a semi-purified diet ± 2% w/w L-glutamine (ICN, Montreal, PQ). The diets were isonitrogenous and isoenergetic: their composition is described in detail in Appendix 1. The fatty acid composition gave a polyunsaturated to saturated fatty acid ratio (P/S) of 0.94 (as determined by gas liquid chromatography). Animals were housed in wire mesh cages in a temperature controlled room, maintained on a 12 hour light/dark cycle and fed *ad libitum*.

After one week of feeding, animals were assigned to one of three activity groups (Baracos, 1989). Rats trained progressively to swim 120 (L) or 240 (H) minutes/day over 21 days (5 days/week) or remained sedentary (S). To minimize the stress response to water, sedentary animals were matched in duration of exposure to (L) and immersed daily in 10 cm of warm (35°C) water. Body weight and food intake were monitored daily.

Rats were killed by CO_2 asphyxiation immediately upon the termination of exercise. Arterial blood was collected by cardiac puncture and the spleen and right and left soleus muscles were removed aseptically. Each experimental group consisted of six animals and all assays were performed on each rat individually.

Succinate Dehydrogenase (SDH) activity:

The soleus muscle was excised, immediately frozen in liquid nitrogen and stored at -70°C for subsequent enzyme analysis. The muscle was homogenized in 0.3 M phosphate buffer (pH 7.2) and analyzed as previously described (Cooperstein et al, 1950). The protein content of each homogenate was determined using a modified Lowry assay (Markwell et al, 1978).

Plasma lactate/pyruvate concentrations:

An aliquot of whole blood was deproteinized in an equal volume of cold 10% (w/v) perchloric acid, centrifuged at 200 g and stored at -20°C for subsequent analysis by spectrophotometric enzymatic methods (Hohorst, 1974; Czok and Lamprecht, 1974).

Splenocyte isolation and metabolism:

Splenocytes were isolated as previously described (Wu et al, 1991b). Triplicate incubations were performed in media containing [U-^{14}C]-glutamine (1 mM, 0.15 µCi/µmol; NEN, Wilmington, DE) ± 4 mM glucose or [U-^{14}C]-glucose (4 mM, 0.15 µCi/µmol; NEN, Wilmington, DE) ± 1 mM glutamine. $^{14}CO_2$ was measured in a Beckman 5000 liquid scintillation counter using modified Bray's solution (Bray, 1960). Ammonia (Bergmeyer and Beutler, 1985), lactate (Hohorst, 1974) and pyruvate (Czok and Lamprecht, 1974) concentrations in the media were determined by

spectrophotometric enzymatic methods. [^{14}C]-glutamate and [^{14}C]-aspartate were separated by ion exchange chromatography (Wu et al, 1991b) and measured in a Beckman 5000 liquid scintillation counter using Scintiverse cocktail (Fisher Scientific, Edmonton, AB). Total ATP concentration in the media was measured using a luciferase assay described by Wu et al (1991b).

Animal protocol [experiment two]:

Forty female Buffalo rats were randomly assigned to two diet groups as in the previous experiment. After one week of feeding, animals were assigned to one of two activity groups, (H) or (S) as described previously. Body weight and food intake were measured on alternate days throughout the study. Rats were killed by CO_2 asphyxiation immediately upon the termination of exercise and at least four hours post-prandially. Arterial blood was collected by cardiac puncture and the spleen and right and left epitrochlearis muscles were removed aseptically.

Plasma amino acid concentrations:

Plasma was separated from arterial blood by centrifugation at 350 g for 20 minutes and stored at -70°C for subsequent analysis. Amino acids were separated using a Varian 5000 high performance liquid chromatograph with a fluorochrome detector (Jones and Gilligan, 1983). Samples were injected onto a Supelcosil 3 micron LC-18 reverse phase column (4.6 X 150 mm; Supelco) and peak area integrations were calculated using a Shimadzu Ezchrom Chromatography Data System.

Natural killer (NK) cytotoxicity:

Natural killer cell activity was determined using a standard 4 hour ^{51}Cr release assay (Field et al, 1991). NK sensitive YAC-1 cells (a gift of Dr A Rabinovitch, Department of Medicine, University of Alberta) were incubated with sodium chromate (^{51}Cr, 150 µCi; Amersham, Oakville, ON) and seeded into 96 well V-bottom microtiter plates. Splenocytes were added in triplicate to the wells to achieve effector:target ratios between 5:1 and 100:1. Following incubation at 37°C, an aliquot (75 µL) of the supernatant was counted in a Beckman 5500 Gamma counter to determine the extent of target cell lysis. Spontaneous release was determined from target cells incubated in the absence of effector cells. Maximum release was determined from detergent lysis of labelled target cells. The percent toxicity was calculated as: % Specific lysis = 100 x [(experimental release-spontaneous release)/(maximum release-spontaneous release)].

[^{3}H]-phenylalanine protein turnover method:

Right and left epitrochlearis muscles were carefully exposed and dissected. Rates of net protein degradation were determined by following the net release of tyrosine into incubation media containing 4 mM glucose, 1mM phenylalanine, 1.0 mM valine, 0.5 mM isoleucine, 0.85 mM leucine and ± 0.1 U/mL insulin (Baracos et al, 1986). Rates of protein synthesis were measured using [^{3}H]-phenylalanine (1.0 mM, 0.05 µCi/mL; NEN, Wilmington, DE) as previously described (Baracos at al, 1986). Paired comparisons (± insulin) were made between contralateral muscles from the same rat.

Statistical analysis:

The effects of diet treatment and exercise were determined by two-way analysis of variance procedures. Natural killer cytotoxicity and net protein degradation (± insulin) were compared among groups by two-way repeated measures analysis of variance. All data is expressed as mean ± standard error (SE).

C. RESULTS

Experiment One:

Body weight and food intake - In male and female rats, there was no significant effect of diet or exercise on final body weight, weight gain or average daily food intake (Table 3-1).

Skeletal muscle enzyme activity - The trained status of exercising animals was verified by enhanced succinate dehydrogenase (SDH) activity in soleus muscle (Figure 3-1). The training effect was greater in the high (H) versus the low (L) exercise group. Plasma lactate and lactate/pyruvate ratios were not different among groups immediately following exercise (Table 3-2).

Splenocyte metabolism - There was no significant effect of diet or exercise on metabolic endproduct production from either glucose (Table 3-3) or glutamine (Table 3-4). No significant gender differences were observed therefore observations were combined. The major endproducts of glucose metabolism were lactate and pyruvate. Glutamine primarily underwent partial oxidation to glutamate, aspartate and ammonia. Less than 10% of glucose and less than 25% of glutamine utilized was recovered as CO_2 (data not shown). The total concentration of ATP in the media (2.35±0.06 nmol/2h/10^6cells, n=36) was not different among groups.

Experiment Two:

Body weight and food intake - Initial and final body weights were not different among groups (Table 3-5). A progressive decrease in voluntary food intake per kilogram

body weight was observed in all groups (Figure 3-3). Spleens from exercising animals weighed less ($p<0.01$) than those from sedentary controls (Table 3-5), however the average number of cells per spleen was not different ($242 \pm 11 \times 10^6$, $n=40$). It is reasonable to assume that exercise induces contraction of the spleen and expulsion of red blood cells into the circulation.

Plasma amino acid concentrations - Amino acid concentrations in arterial plasma were described in detail (Table 3-6). Exercise significantly decreased plasma glutamate, threonine, alanine and ornithine concentrations and significantly increased plasma lysine concentrations (Figure 3-4). Glutamine supplementation significantly ($p<0.05$) reduced the concentration of aspartate and serine in plasma (Table 3-6). Plasma glycine concentrations were three-fold higher ($p<0.001$) in the non-glutamine supplemented diet group.

Natural killer cytotoxicity - Neither diet nor exercise significantly altered natural killer cytolytic activity in splenocytes as determined by % specific lysis of ^{51}Cr-labelled YAC-1 cells at any of the effector:target ratios measured (Figure 3-5).

Net protein degradation in skeletal muscle - Net protein degradation in the epitrochlearis muscle incubated in the absence of insulin was not significantly different among groups (Figure 3-6). Insulin (0.1 U/mL) reduced ($p<0.001$) net proteolysis in the epitrochlearis muscle in all groups, however, the resulting rates did not differ significantly among experimental groups.

D. DISCUSSION

Body weight and food intake:

Exercising animals did not increase their average daily food intake to compensate for increased expenditure due to exercise. The ability of these animals to sustain growth could reflect an alteration in one or more components of total energy expenditure, although the possibility was not investigated in the current study. Long-term energy balance studies in male rats reveal that exercise causes a marked decline in energy intake that contributes, in association with increased energy expenditure, to slowed growth; in female rats, exercise does not affect food intake and lower energy gains result solely from increased expenditure (Richard and Rivest, 1989). Body weight did not differ between groups, however the effect of exercise on total body composition was not measured in this study.

Plasma amino acid concentrations:

Dietary L-glutamine supplementation did not result in significantly increased plasma concentrations of this amino acid. It is likely that most or all of the glutamine supplied by the supplemented diet was utilized by cells of the small intestine. Glutamine is the major respiratory fuel for enterocytes and contributes substantially to the energy required for active transport of nutrients and electrolytes from the lumen (reviewed in Koracevic and McGivan, 1983). The intestinal mucosa also exhibits a high rate of protein synthesis and cell turnover and glutamine may be an important energy source for these processes. The inability to transport dietary amino acids across the lumen was not likely a factor since a three-fold increase in glycine concentration was observed in the non-glutamine supplemented group.

Exercise did not result in significantly decreased plasma glutamine concentrations. These results are not in agreement with data from human subjects (Rennie et al, 1981) and animals (Parry-Billings et al, 1990) that suggests plasma glutamine levels decrease following exercise of high intensity and long duration.

Glucose and glutamine metabolism in splenocytes:

The results of this study suggest no change in the rate of glucose or glutamine metabolism arising from exercise or dietary supplementation. Although the metabolism of glucose, glutamine and other substrates has been well characterized in splenocytes of normal and diabetic rats (Wu et al, 1991a), little is known about their metabolism during exercise. Clearly, the importance of fatty acid and lactate metabolism in these cells remains to be determined particularly when exercise markedly increases plasma concentrations of these substrates.

Natural killer cell cytotoxicity:

In the current study, acute exercise had no effect on natural killer cell activity. This result was not completely unexpected. It is possible that training attenuated the exercise-associated stress response in swimming animals such that it was not different from that experienced by sedentary animals immersed in water. This explanation is supported by the low relative intensity of the swimming exercise as indicated by the lactate/pyruvate ratios. The stress associated with CO_2 asphyxiation at the time of sacrifice was assumed to be the same for all groups.

Exercise-stress impairs lymphocyte proliferation to antigens and mitogens (Hoffman-Goetz et al, 1986; Hoffman-Goetz et al, 1988) and decreases plasma immunoglobulin concentrations and macrophage reactivity (Keller et al, 1984). The underlying physiological mechanism(s) has not been clarified, although the role of

catecholamines has been suggested (Kappel et al, 1991). Previous studies have demonstrated an immediate and transient increase in the number and activity of natural killer cells in peripheral blood (Pedersen et al, 1988) and the spleen (Simpson and Hoffman-Goetz, 1990) following acute submaximal or maximal exercise. The intravenous infusion of epinephrine to achieve plasma concentrations observed during exercise has been shown to mimic these effects (Kappel et al, 1991).

Net protein degradation in muscle:

No effect of exercise on net protein degradation in the isolated epitrochlearis muscle was observed in the present study, although exercise has previously been associated with increased whole body catabolism of protein and amino acids (Rennie et al, 1981). Isolated muscle preparations are in a catabolic state, however, muscles maintained *in vitro* under optimal nutritional conditions respond to the various regulatory factors that affect protein turnover *in vivo* (Baracos et al, 1986). Losses arising from catabolism generally represent only a small proportion of the total protein mass (Baracos et al, 1989).

Insulin has profound anabolic effects on a variety of tissues and stimulates amino acid transport into muscle by activating a sodium-dependent transport mechanism. The observed decrease in tyrosine release when insulin was added to the media was probably accounted for by the ability of insulin to simultaneously increase protein synthesis and inhibit protein degradation. Both processes appear to be of comparable import in improving nitrogen balance in isolated muscle (Goldberg et al, 1980). This possibility was not investigated in the current experiment.

A positive relationship between protein synthetic rate and intramuscular glutamine concentration exists in the presence and absence of insulin in perfused muscle (MacLennan et al, 1987). If this effect can be extrapolated to isolated muscle, the absence of a diet effect on net protein degradation in this experiment implies that dietary glutamine supplementation had no significant effects on intramuscular glutamine concentrations.

Overall, the present study demonstrates no effect of exercise or dietary L-glutamine supplementation on splenocyte metabolism, splenic NK activity or net protein degradation in muscle. Inasmuch as low intensity swimming exercise did not decrease plasma glutamine concentration or enhance splenocyte metabolism, no increased requirement for glutamine by immune cells during or following exercise was evident.

Table 3-1: Body weight and food intake of male and female rats within activity groups**

		Initial Weight (g)	Final Weight (g)	Weight Change (g)	Intake (g/kg/day)
Exercise (H)	♂ (6)	287.9 ± 5.6*	364.5 ± 3.5	76.6 ± 3.7	58.5 ± 1.0
	♀ (6)	190.1 ± 4.2	222.9 ± 6.9	32.7 ± 3.0	59.3 ± 0.6
Exercise (L)	♂ (6)	285.1 ± 6.8	362.9 ± 5.9	77.8 ± 5.5	58.1 ± 1.5
	♀ (6)	189.3 ± 2.1	224.1 ± 4.6	34.6 ± 3.9	59.1 ± 1.3
Sedentary (S)	♂ (6)	264.0 ± 7.4	346.1 ± 9.7	82.1 ± 6.4	61.5 ± 1.2
	♀ (6)	187.3 ± 2.4	222.6 ± 6.8	35.3 ± 4.5	58.0 ± 1.3

*Values are means ± SE. The number of animals in each group is indicated in parentheses. **No significant effect of diet by two-way analysis of variance procedure, therefore groups were combined.

Table 3-2: Plasma lactate concentrations in response to diet and exercise*

Group	Plasma Lactate (mmol/L)	L/P ratio (Lactate:Pyruvate)
HGLN+ (6)	2.72 ± 0.23	6.73 ± 1.03
LGLN+ (6)	3.14 ± 0.53	9.22 ± 1.30
SGLN+ (6)	2.83 ± 0.45	8.24 ± 1.54
HGLN– (6)	2.67 ± 0.15	9.95 ± 1.65
LGLN– (6)	2.84 ± 0.36	9.00 ± 1.04
SGLN– (6)	2.19 ± 0.13	6.43 ± 0.43

*NS, two-way analysis of variance procedure. The number of animals in each group is indicated in parentheses. Abbreviations used: H, high; L, low; S, sedentary; Gln+, glutamine supplemented diet; Gln– , non-glutamine supplemented diet.

Table 3-3: Endproducts of glucose metabolism in freshly isolated splenocytes*

Diet Group	Glutamine +			Glutamine –		
Activity Level	High (H)	Low (L)	Sedentary (S)	High (H)	Low (L)	Sedentary (S)
Metabolite	nmol/2h/10^6 cells					
$^{14}CO_2$	4.68 ± 0.46 (5)	4.74 ± 0.39 (7)	4.70 ± 0.42 (6)	4.64 ± 0.46 (5)	4.82 ± 0.39 (7)	4.93 ± 0.46 (5)
Lactate	20.87 ± 1.44 (5)	21.42 ± 1.44 (5)	21.25 ± 1.61 (4)	19.18 ± 1.61 (4)	22.69 ± 1.31 (6)	21.35 ± 1.31 (6)
Pyruvate	17.51 ± 2.01 (5)	19.62 ± 1.70 (7)	19.89 ± 1.84 (6)	22.41 ± 2.25 (4)	18.96 ± 1.84 (6)	18.58 ± 2.25 (4)

Values are means ± SE (n). Incubation media contained 4 mM [U-^{14}C] glucose + 1 mM glutamine. *NS, two-way analysis of variance procedure.

TABLE 3-4: Endproducts of glutamine metabolism in freshly isolated splenocytes*

Diet Group	Glutamine +			Glutamine –		
Activity Level	High (H)	Low (L)	Sedentary (S)	High (H)	Low (L)	Sedentary (S)
Metabolite	nmol/2h/10^6 cells					
$^{14}CO_2$	5.05 ± 0.56 (5)	4.59 ± 0.56 (5)	4.60 ± 0.56 (5)	4.56 ± 0.56 (5)	5.11 ± 0.51 (6)	4.47 ± 0.56 (5)
[^{14}C]-Glutamate	3.41 ± 0.48 (4)	3.66 ± 0.36 (7)	4.18 ± 0.43 (5)	3.14 ± 0.48 (4)	3.44 ± 0.48 (4)	3.29 ± 0.43 (5)
[^{14}C]-Aspartate	0.49 ± 0.05 (6)	0.52 ± 0.05 (8)	0.54 ± 0.05 (6)	0.47 ± 0.05 (6)	0.48 ± 0.05 (6)	0.53 ± 0.05 (5)

Values are means ± SE (n). Incubation media contained 1 mM [U-^{14}C] glutamine + 4 mM glucose. *NS, two-way analysis of variance procedure.

Table 3-5: Animal and spleen weights

Group	Initial Weight (g)	Final Weight (g)	Spleen Weight (g)
HGLN+ (10)	178.0 ± 4.3	218.0 ± 4.6	0.392 ± 0.012*
SGLN+ (10)	176.1 ± 2.6	217.1 ± 4.0	0.425 ± 0.011
HGLN– (10)	179.4 ± 3.4	213.8 ± 3.6	0.395 ± 0.012*
SGLN– (10)	180.3 ± 4.2	223.5 ± 4.7	0.418 ± 0.011

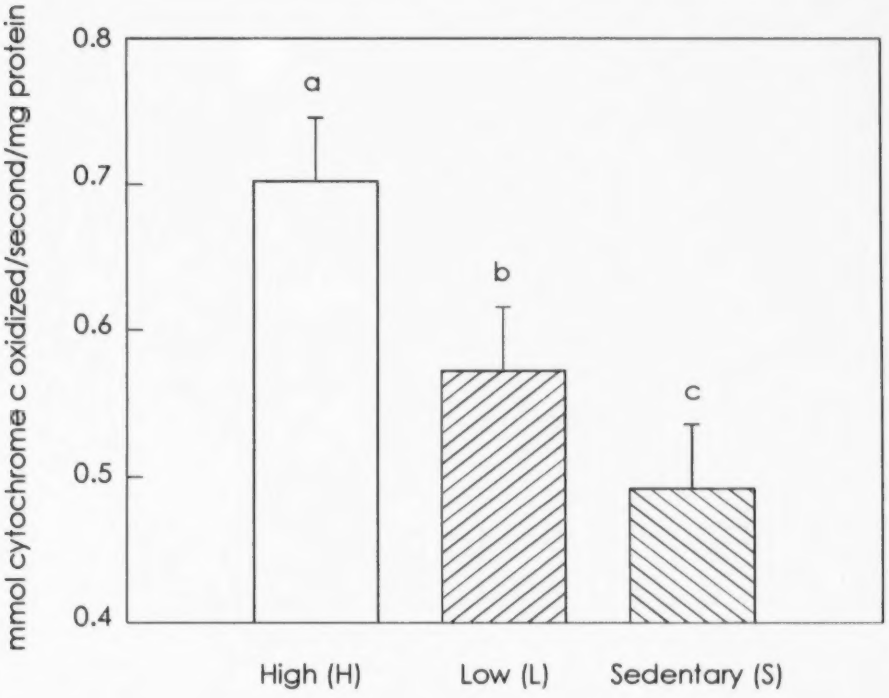
Values are means ± SE. The number of animals in each group is indicated in parentheses. *$p<0.01$ exercise, two-way analysis of variance procedure. Abbreviations used: H, high; S, sedentary; Gln+, glutamine-supplemented diet; Gln– , non-glutamine supplemented diet.

Table 3-6: Amino acid concentrations in arterial plasma

Diet Group	Glutamine+		Glutamine-		Significance**	
Activity Level	Exercise (H)	Sedentary (S)	Exercise (H)	Sedentary (S)	Diet	Exercise
Amino Acid	nmol/mL					
Alanine	273.2 ± 19.5 (8)*	342.3 ± 18.4 (9)	265.6 ± 17.4 (10)	338.4 ± 16.6 (11)	NS	0.001
Aspartate	12.3 ± 1.1 (9)	10.8 ± 1.1 (9)	13.1 ± 1.0 (10)	15.1 ± 1.0 (10)	0.05	NS
Glutamate	98.7 ± 15.6 (8)	159.4 ± 13.9 (10)	115.8 ± 13.9 (10)	161.2 ± 13.3 (11)	NS	0.001
Glutamine	546.9 ± 26.5 (8)	564.7 ± 25.0 (9)	525.7 ± 25.0 (9)	554.7 ± 23.7 (10)	NS	NS
Glycine	125.5 ± 20.7 (9)	112.1 ± 19.7 (10)	324.6 ± 19.7 (10)	300.7 ± 19.7 (10)	0.001	NS
Isoleucine	129.8 ± 6.1 (9)	126.8 ± 5.8 (10)	118.7 ± 5.7 (10)	117.5 ± 5.5 (11)	NS	NS
Leucine	202.6 ± 10.5 (9)	190.3 ± 9.9 (10)	198.9 ± 9.9 (10)	183.2 ± 9.5 (11)	NS	NS
Lysine	602.0 ± 24.0 (8)	532.7 ± 21.5 (10)	540.4 ± 21.5 (10)	512.0 ± 20.5 (11)	NS	0.05
Ornithine	114.0 ± 6.8 (8)	126.6 ± 6.4 (9)	103.2 ± 6.1 (10)	116.0 ± 5.8 (11)	NS	0.05
Phenylalanine	71.1 ± 6.4 (9)	69.5 ± 6.4 (9)	79.3 ± 6.1 (10)	69.2 ± 5.8 (11)	NS	NS
Serine	212.0 ± 18.9 (8)	214.2 ± 16.9 (10)	291.6 ± 16.9 (10)	328.6 ± 16.1 (11)	0.001	NS
Threonine	283.3 ± 19.6 (9)	340.4 ± 18.6 (10)	264.5 ± 18.6 (10)	292.9 ± 17.7 (11)	NS	0.05
Tyrosine	82.3 ± 5.5 (9)	79.8 ± 5.2 (10)	88.2 ± 5.2 (10)	80.6 ± 5.0 (11)	NS	NS
Valine	253.2 ± 13.2 (9)	236.9 ± 12.5 (10)	249.8 ± 12.5 (10)	231.5 ± 11.9 (11)	NS	NS

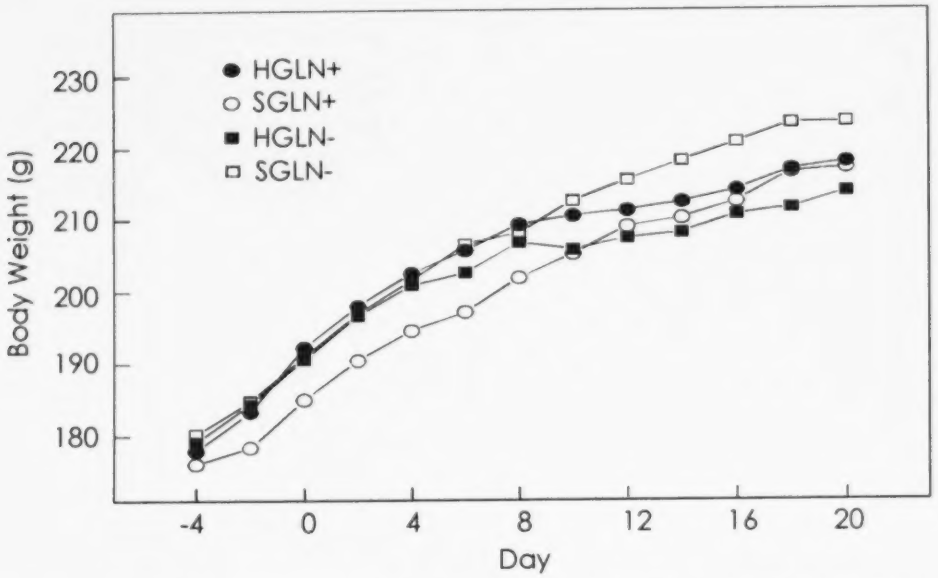
*Values are means ± SE (n). **Significant effects by two-way analysis of variance procedure; no significant interaction was found for any amino acid tested.

Figure 3-1: Succinate dehydrogenase (SDH) activity in the soleus muscle of rats following exercise training*



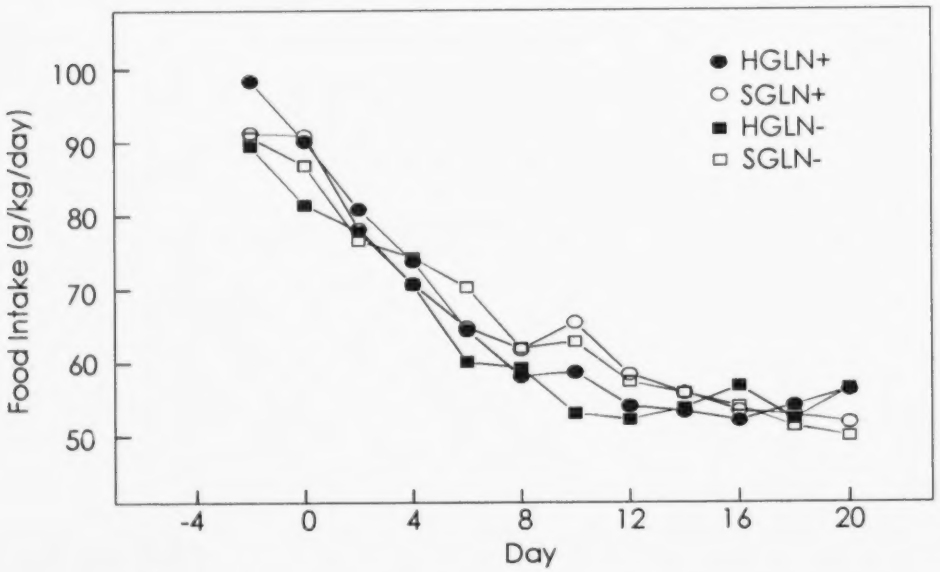
* No significant effect of diet by two-way analysis of variance procedure, therefore diet groups were combined. Comparison by one-way analysis of variance procedure; values without a common letter are significantly different ($p<0.05$).

Figure 3-2: Effect of diet and exercise on total body weight gain in female rats*



*NS, two-way repeated measures analysis of variance procedure, n=10 animals/group.

Figure 3-3: Effect of diet and exercise on average daily food intake in female rats*



*NS, two-way repeated measures analysis of variance procedure, n=10 animals/group.

Figure 3-4: Effect of swim-training on arterial plasma amino acid concentrations in female rats

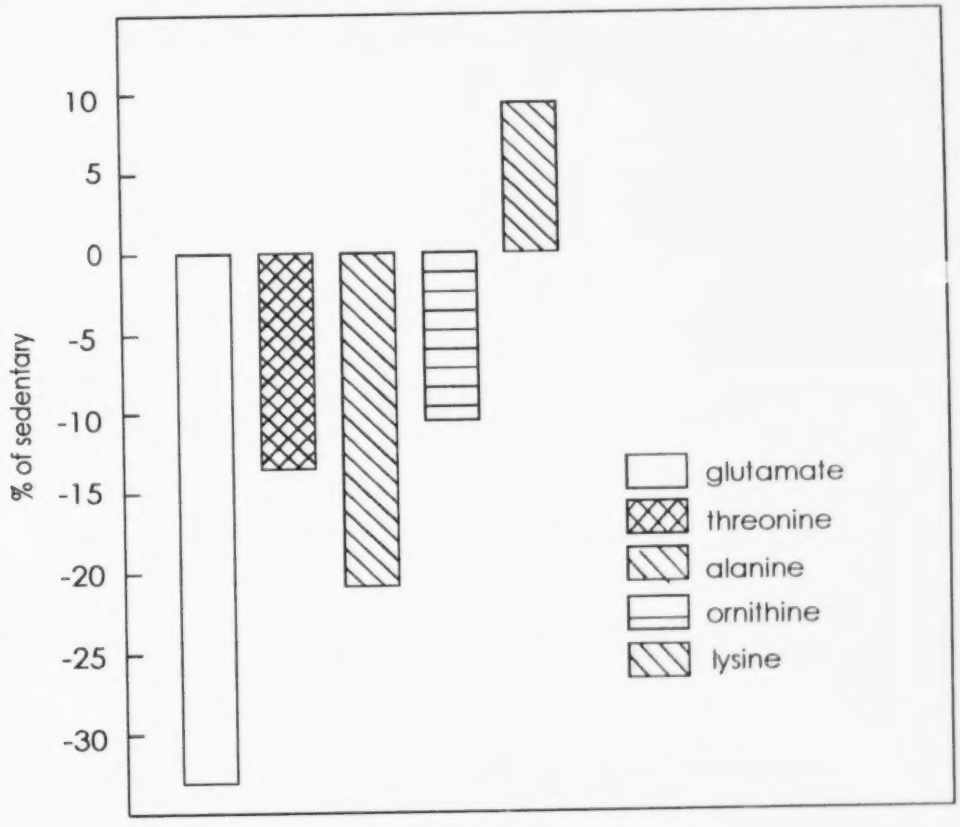
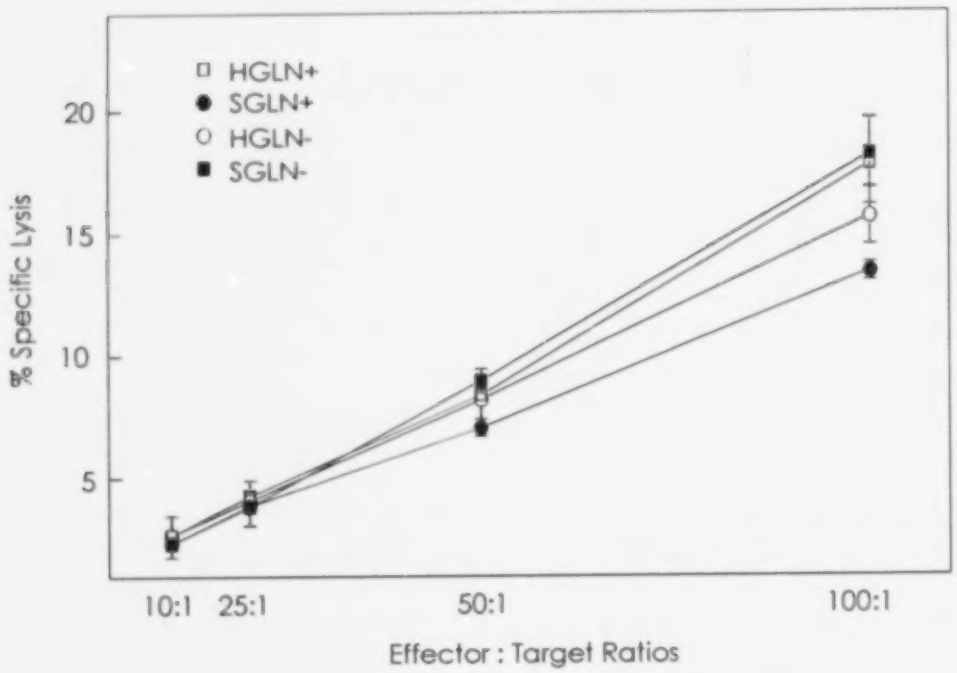
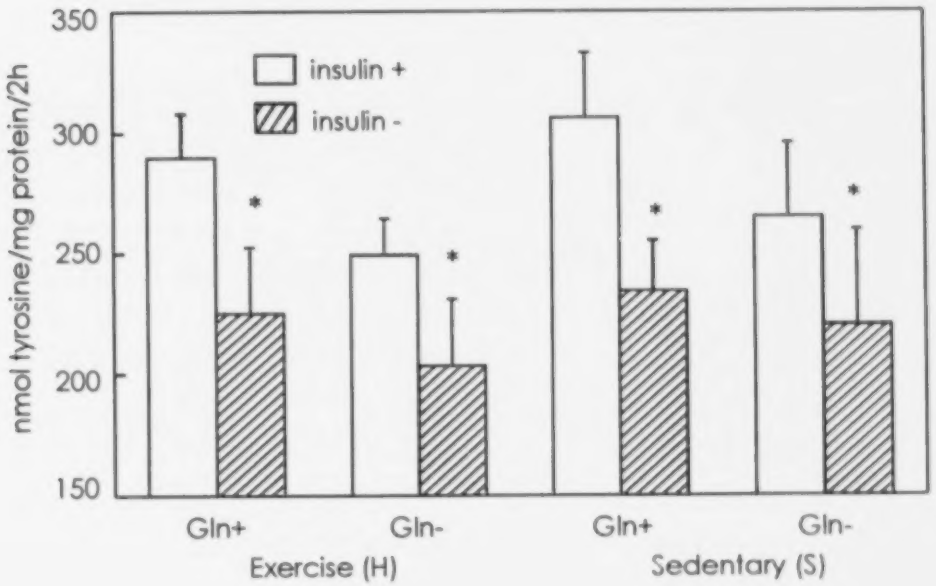


Figure 3-5: Effect of diet and exercise on splenic natural killer cytolytic activity in female rats*



*NS, two-way repeated measures analysis of variance procedure, n=10 animals/group.

Figure 3-6: Effect of insulin on net protein degradation in rat epitrochlearis muscles incubated in the absence of tyrosine



Results, given as group means± SE, were analyzed by two-way repeated measures analysis of variance. * indicates a significant ($p<0.001$) effect of insulin within an exercise/diet group, n=10 animals/group.

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4. DIETARY L-GLUTAMINE SUPPLEMENTATION BUT NOT EXERCISE INHIBITS THE GROWTH OF THE MORRIS HEPATOMA 7777 IN RATS

A. INTRODUCTION

Numerous animal models clearly demonstrate that tumor growth and metastasis are stimulated during periods of nutritional support (Torosian, 1992). Although nutrient administration could selectively feed the tumor and potentiate its growth, the possibility of improving host nutritional status and immunocompetence must also be considered. Recently, the ability of a glutamine-enriched enteral diet to replete host glutamine stores and support muscle glutamine metabolism without stimulating tumor growth was demonstrated in rats (Klimberg et al, 1990).

Exercise can also alter the course of malignancy in experimental animals. Depending on the tumor model, the phase of neoplasia and the nature and timing of the exercise, physical activity has the potential to either inhibit or promote tumorigenesis (reviewed in MacNeil and Hoffman-Goetz, 1993). Recently, a significant reduction in the size and protein content of a rat hepatoma was observed regardless of whether swimming exercise was initiated prior to or immediately following transplantation of the tumor (Baracos, 1989). The mechanisms associated with the inhibition of growth were not evident, however. The objective of the present study was to examine concurrently the effects of dietary L-glutamine supplementation and exercise on specific parameters of immune function and growth of the Morris hepatoma 7777. The temporal relationship between tumor development and immune responsiveness was also examined.

B. MATERIALS AND METHODS

Animal protocol [experiment three]:

To evaluate the effects of tumor progression on immune function, twenty-five female Buffalo rats were housed individually, fed the non-glutamine supplemented diet (GLN-) and remained sedentary. After one week of feeding, twenty rats received two 20 µL subcutaneous injections of finely chopped Morris hepatoma 7777, one on each flank (Baracos, 1989). The Morris hepatoma is a poorly differentiated and rapidly growing transplantable tumor (Fields et al, 1981). It is locally aggressive, metastasizes rarely and results in death of the host four to five weeks following implantation (Fields et al, 1981). Tumor growth was allowed to progress for two (t=2

wks, n=10) or three (t=3 wks, n=10) weeks. Body weight and food intake were recorded on alternate days throughout the study. Rats were killed by CO_2 asphyxiation at the end of the dark cycle and the tumors carefully excised. Individual (data not shown) and total tumor weights were recorded for each animal. Five rats served as non-tumor-bearing controls (t=0 wks). Arterial blood was collected by cardiac puncture and the spleen was removed aseptically.

Animal protocol [experiment four]:

Thirty-five female Buffalo rats were randomly assigned to two diet (GLN+ or GLN-) groups as described previously (Chapter 3). After one week of feeding, all rats received two 20 µL subcutaneous hindlimb injections of finely chopped tumor (Baracos, 1989) and were assigned to one of two activity groups, (H) or (S). Rats trained progressively to swim 180 minutes a day (H) over 14 days (6 days/week) or remained sedentary (S). Sedentary animals were matched in duration of exposure and immersed daily in 10 cm of warm water (35°C). Body weight and food intake were monitored daily throughout the study. Twenty-four hours following the last bout of exercise, rats were killed at the end of the dark cycle by CO_2 asphyxiation. Arterial blood, tumors, spleen and soleus muscles were removed as previously described. All assays were performed on each rat individually.

Animal protocol [experiment five]:

The protocol described in experiment four was repeated using twenty-four female Buffalo rats. Each experimental group consisted of six animals and all assays were performed on each rat individually. Data was combined where means were not statistically different between experiments.

Plasma amino acid concentrations:

Plasma was separated from arterial blood by centrifugation at 350 g for 20 minutes and stored at -70°C for subsequent analysis. Amino acids were separated using a Varian 5000 high performance liquid chromatograph with a fluorochrome detector (Jones and Gilligan, 1983). Samples were injected onto a Supelcosil 3 micron LC-18 reverse phase column (4.6 X 150 mm; Supelco) and peak area integrations were calculated using a Shimadzu Ezchrom Chromatography Data System.

Natural killer (NK) cytotoxicity:

Natural killer cell activity was determined using a standard 4 hour ^{51}Cr release assay (Field et al, 1991). NK sensitive YAC-1 cells (a gift of Dr A Rabinovitch, Department of Medicine, University of Alberta) were incubated with sodium chromate (^{51}Cr, 150 µCi; Amersham, Oakville, ON) and seeded into 96 well V-bottom microtiter plates. Splenocytes were added in triplicate to the wells to achieve effector:target ratios

between 5:1 and 100:1. Following incubation at 37°C, an aliquot (75 µL) of the supernatant was counted in a Beckman 5500 Gamma counter to determine the extent of target cell lysis. Spontaneous release was determined from target cells incubated in the absence of effector cells. Maximum release was determined from detergent lysis of labelled target cells. The percent cytotoxicity was calculated as: % Specific Lysis = 100 x [(experimental release-spontaneous release)/(maximum release-spontaneous release)].

Splenocyte response to mitogen stimulation:

Splenocytes were cultured in 96 well microtiter plates at a density of $5x10^5$ cells/well with Concanavalin A (Con A, 5 µg/mL; ICN, Montreal, PQ) or phorbol myristate acetate (PMA, 30 ng/mL; ICN, Montreal, PQ) plus ionomycin (Iono, 0.75 nmol/mL; Sigma Chemicals, St Louis, MO) as previously described (Field et al, 1991). Optimal mitogen dose and length of incubation were previously determined (Figure A-1). After 36 or 54 hours in culture at 37°C, 1 µCi [^{3}H]-thymidine (Amersham, Oakville, ON) was added to each well. Eighteen hours later, the cultures were collected on glass fibre filters using a multiwell harvester (Skatron, Norway). [^{3}H]-thymidine incorporation was measured in a Beckman 5000 liquid scintillation counter. All assays were performed in quadruplicate on individual rats. Stimulation indices were calculated for each mitogen as: SI = [amount of [^{3}H]-thymidine (counts/minute) incorporated by the stimulated cells - amount of [^{3}H]-thymidine (counts/minute) incorporated by the unstimulated cells]/[amount of [^{3}H]-thymidine (counts/minute) incorporated by the unstimulated cells].

The media used for all assays was RPMI 1640 (Fisher Scientific, Edmonton, AB) containing penicillin (100 U/mL), streptomycin (100 µg/mL), amphotericin B (conc), N-2-hyroxyethylpiperazine-N'-2-ethanesulfonic acid buffer solution (HEPES, 25 mM), glutamine (4 mM), heat-inactivated fetal bovine serum (FBS, 5% v/v; ICN, Montreal, PQ) and 2-mercaptoethanol (2.5 µM).

Splenocyte isolation and metabolism:

Splenocytes were isolated as previously described (Wu et al, 1991). Triplicate incubations were performed in media containing [U-^{14}C]-glutamine (1 mM, 0.15 µCi/µmol; NEN, Wilmington, DE) ± 4 mM glucose or [U-^{14}C]-glucose (4 mM, 0.15 µCi/µmol; NEN, Wilmington, DE) ± 1 mM glutamine. $^{14}CO_2$ was measured in a Beckman 5000 liquid scintillation counter using modified Bray's solution (Bray, 1960). Ammonia (Bergmeyer and Beutler, 1985), lactate (Hohorst, 1974) and pyruvate (Czok and Lamprecht, 1974) concentrations in the media were determined by spectrophotometric enzymatic methods. [^{14}C]-glutamate and [^{14}C]-aspartate were

separated by ion exchange chromatography (Wu et al, 1991) and measured in a Beckman 5000 liquid scintillation counter using Scintiverse cocktail (Fisher Scientific, Edmonton, AB).

Mononuclear Cell Phenotyping:

Lymphocyte subsets from spleen were detected by direct immunofluorescence (Field et al, 1991). Monoclonal antibodies (OX8, OX12, OX19, OX42, W3/25, 3.2.3; gifts from Dr A Rabinovitch, Department of Medicine, University of Alberta except 3.2.3 which was a gift from Dr P Poussier, St Michael's Hospital, University of Toronto) bound to cell surface receptors were conjugated to fluorescein isothiocyanate (FITC, Cappel goat anti-IgG mouse; Organon Teknika, Scarborough, ON). After fixation with 1% paraformaldehyde, labelled cells were analyzed by flow cytometry (FACScan, Becton Dickson).

Statistical analysis:

The effects of diet treatment and exercise were determined by two-way analysis of variance procedures. Natural killer cytotoxicity was compared across effector:target ratios by two-way repeated measures analysis of variance. All data is expressed as mean ± standard error (SE).

C. *RESULTS*

Experiment Three:

Body weight, spleen weight, tumor weight and food intake - Initial and final body weight and spleen weight were not significantly different among groups (Table 4-1). Tumor-bearing animals were studied when tumor burden was small (1.4 ± 0.1 % final body weight, t=2 weeks) or large (6.1 ± 0.5 % final body weight, t=3 weeks). Rate of body weight gain (Figure 4-1) and average daily food intake (Figure 4-2) are shown. Body weight increased progressively through the experiment while food intake per kilogram of body weight decreased.

Plasma amino acid concentrations - Amino acid concentrations in arterial plasma were described in detail (Table 4-2). Plasma leucine, isoleucine, valine and tyrosine concentrations were depressed ($p<0.05$) three weeks following tumor implantation relative to controls (t=0 weeks). At two weeks the concentration of all of these amino acids did not differ from controls. Plasma aspartate concentrations increased ($p<0.05$) at two weeks but were not different from control levels at three weeks.

Natural killer (NK) cytotoxicity - Splenic NK cytotoxicity was significantly ($p<0.001$) depressed two weeks following tumor implantation but increased ($p<0.001$) above control values at three weeks (Figure 4-3). This effect was apparent when analyzed

across all effector:target ratios measured. Interassay variation is low; the response of control animals (t=0 weeks) was not different from that reported for healthy animals in the previous experiment (Chapter 3).

Splenocyte response to mitogen stimulation - Incubation with the mitogen Con A and the mixture of PMA + Iono significantly increased ^{3}[H]-thymidine incorporation by immune cells from control (t=0 weeks) and tumor-bearing (t=2 weeks, t=3 weeks) animals at both 48 and 72 hours (Figure 4-4). A higher response to Con A was observed in all groups at 72 hours and the maximum response to PMA + Iono in t=0 weeks and t=2 weeks appeared to occur at 48 hours since [^{3}H]-thymidine incorporation decreased at 72 hours. For t=3 weeks, the response to PMA + Iono was higher at 72 than 48 hours. A decreased ($p<0.05$) response to Con A and PMA + Iono was observed in cells from tumor-bearing animals relative to controls after 48 hours (t=3 weeks) and 72 hours (t=2 weeks and t=3 weeks) in culture. For Con A, the response was lower ($p<0.05$) at both time points in cells from t=3 versus t=2 weeks. Because the unstimulated (control) incorporation increased in cells from t=2 weeks at 48 hours and decreased in cells from t=3 weeks at 72 hours, stimulation indices were also expressed (Table 4-3).

Splenocyte metabolism - Tumor-bearing significantly ($p<0.01$) reduced carbon dioxide and pyruvate production from glucose (Table 4-4) by splenocytes. The addition of glutamine to the media decreased ($p<0.01$) CO_2 production in all groups and decreased ($p<0.01$) lactate production in the control group; pyruvate production increased only in animals with a large tumor burden. Tumor-bearing significantly ($p<0.01$) reduced CO_2 production from glutamine but did not alter the production of glutamate, aspartate or ammonia (Table 4-5). The addition of glucose to the media reduced ($p<0.01$) $^{14}CO_2$ and aspartate production in all groups; glutamate and ammonia production decreased only in control animals.

<u>*Experiment Four/Five:*</u>

Body weight, spleen weight, tumor weight and food intake - Total body weight (Figure 4-5) and average daily food intake per kilogram body weight (Figure 4-6) were not different among groups following tumor implantation. Final body weight was significantly greater ($p<0.05$) than initial body weight for all groups (Table 4-6, statistics not shown). Exercise reduced ($p<0.01$) spleen weight (Table 4-6) but did not alter the total number of cells per spleen ($249 \pm 8 \times 10^{6}$, n=35). This effect may be related to the contraction of the spleen during exercise and the expulsion of red blood cells into the circulation. Dietary glutamine supplementation decreased ($p<0.001$) both absolute tumor weight and tumor weight expressed as a % of total body weight (Table 4-6).

Plasma amino acid concentrations - Plasma amino acid concentrations were described in detail (Table 4-7). Glutamine supplementation increased ($p<0.001$) plasma glutamate, threonine, valine and leucine and decreased plasma serine concentrations (Figure 4-7). Plasma glycine concentrations were three-fold higher ($p<0.001$) in the non-glutamine supplemented group. Exercise training decreased plasma aspartate, threonine, valine and leucine concentrations (Figure 4-8).

Natural killer cytotoxicity - Neither diet nor exercise had a significant effect on splenic NK cell activity at the effector:target ratios measured (Figure 4-9).

Splenocyte response to mitogen stimulation - [^{3}H]-thymidine incorporation in unstimulated (control) cells was not different among groups (Figure 4-10) at both 48 and 72 hours. There was a low response to Con A at 48 hours in all groups and the maximum response was observed at 72 hours. Cells stimulated with PMA + Iono responded equally at 48 and 72 hours. The response to Con A was greater ($p<0.05$) at 72 hours in trained (H) animals relative to sedentary (S) controls. The response to PMA + Iono was greater ($p<0.005$) in trained animals at both 48 and 72 hours. Stimulation indices are expressed in Table 4-8.

Mononuclear cell populations in spleen - The relative proportion of mononuclear cell phenotypes identified using anti-rat monoclonal antibodies measured in spleen were not different among groups (Table 4-9).

D. DISCUSSION

Tumor progression:

When tumor growth is allowed to progress in this animal model, central necrosis within the tumor mass occurs, confounding the relationship of measured tumor size and viable, metabolically active tissue. Thus, the duration of the study was long enough to reveal differences produced by glutamine supplementation while maintaining the accuracy of physical tumor measurements and preventing the development of severe cachexia in the host.

Exercise and diet effects on tumor growth:

Exercise did not inhibit the growth of the Morris hepatoma 7777 in this animal model. Previous reports of exercise-induced inhibition (Baracos, 1989) may have been related to a relative energy deficit in the chow-fed animals that was not present in animals fed a high fat diet. In other animal models, protein-calorie restriction is associated with a reduction in spontaneous tumorigenesis, inhibition of primary tumor growth and decreased establishment of transplanted tumors (reviewed in Torosian, 1992). Forty-percent calorie restriction inhibited the growth of DMBA-induced

mammary and DMH-induced colon tumors even when the diet contained twice as much fat as the control diet (Kritchevsky, 1990); a similar inhibition of tumor growth was achieved when rats fed *ad libitum* were subjected to high intensity treadmill exercise. Moreover, decreased total fat and energy intake, increased energy expenditure due to exercise and a substitution of n-3 fatty acids for n-6 fatty acids has been shown to suppress pancreatic carcinogenesis in rats (Roebuck, 1992). In the current study, it is also possible that changes in membrane phospholipid composition resulting from dietary fat manipulation may have altered the responsiveness of the tumor tissue to the antitumor defence mechanisms mobilized by exercise training.

Dietary L-glutamine supplementation significantly decreased tumor growth in the present study, although the mechanisms for this effect are not clear. Theoretically, competition for glutamine between the host and tumor would be less if lymphocytes could derive energy from other circulating metabolites in plasma. Although the importance of other metabolites during exercise and in the tumor-bearing host has not been systematically investigated, it is known that citrulline, a byproduct of glutamine oxidation in the small intestine, can be used by other tissues for arginine biosynthesis. Indeed, the addition of arginine to basal diets has been shown to result in protection against both the growth of transplantable tumors and the tumorigenicity of carcinogens by upregulating existing host immune responses (Daly et al, 1990).

Plasma amino acid concentrations:

Plasma glutamine concentrations were not significantly increased in animals fed the glutamine supplemented diet. The supplemental glutamine was likely utilized by the small intestine, skeletal muscle or the tumor. Previous studies have described significant increases in muscle glutamine concentration and hindquarter glutamine efflux without concurrent increases in plasma concentrations in glutamine-fed tumor-bearing rats (Klimberg et al, 1990). These effects were best explained by a stimulation of *de novo* intracellular glutamine synthesis in muscle and/or by augmented uptake of glutamine by skeletal muscle during the post-prandial state (Klimberg et al, 1990).

Plasma glutamine concentrations were not significantly depressed in animals fed the non-glutamine supplemented diet, possibly because of increased glutamine release by the liver or muscle or decreased utilization by the gastrointestinal tract. Depressed plasma glutamine levels in tumor-bearing rats reported by other researchers could be related to the decrease in voluntary food intake associated with malignant disease. Moyer and Pitot (1974) reported lower weight gains and depressed glutamine concentrations in tumor-bearing rats relative to control animals, however, the controls were not pair-fed. When body weight of control rats is reduced

by restricted feeding to match carcass weights of tumor-bearing animals, levels of hydroxyproline, threonine, asparagine, proline, alanine, citrulline, valine, leucine, isoleucine, tyrosine, ornithine, lysine and arginine reportedly fall (Chance et al, 1988), thus decreases in amino acid concentrations in tumor-bearing rats could be related to anorexia. It is difficult to completely explain changes in plasma concentration arising from dietary manipulation or exercise because of the complex interrelationships that exist between the rate of uptake and synthesis of the various amino acids by the tumor and by host tissues.

Splenocyte metabolism:

Glucose and glutamine oxidation to carbon dioxide was significantly lower in splenocytes from tumor-bearing rats, however, the production of major metabolic endproducts from these substrates (lactate, glutamate and ammonia) was not altered. Decreased rates of cellular metabolism relative to control animals suggests a downregulation of the activity of the immune system in response to the presence of the tumor. These results are supported by decreased rates of [^{3}H]-thymidine incorporation into splenocytes following mitogen stimulation. Stimulated cells of the immune system have high energy requirements and accelerated rates of protein and nucleic acid biosynthesis (reviewed in Lacey and Wilmore, 1990). The high rates of partial oxidation in these cells is thought to indicate an increased need for the biosynthetic intermediates of glycolysis and glutaminolysis.

Splenic response to mitogen stimulation:

Unstimulated splenocytes for tumor-bearing rats (t=2 wks) displayed significantly greater [^{3}H]-thymidine incorporation than splenocytes from control animals (Figure 4-4). This observation suggests stimulation of the immune system in animals with a small tumor burden. The ability of splenocytes to respond to stimulation by Con A or PMA + Iono suggests that cell-mediated immune function was not completely obliterated in tumor-bearing animals. It is noteworthy, however, that responses were most severely depressed in animals with a large tumor burden.

Mitogen-induced lymphocyte transformation provides a useful model for amplifying the antigen-specific responses that occur *in vivo*. Stimulation with Con A, a T-cell mitogen, requires the participation of specialized accessory cells and induces the production of soluble mediators and thus is believed to reasonably portray the state of immune responsiveness *in vivo*. The decreased proliferative response of splenocytes from tumor-bearing animals to this mitogen might indicate an abnormality in the binding of the mitogen to the cell membrane receptor, altered accessory cell function or a change in intracellular signal transduction (Field et al,

1990). PMA + Iono stimulates cells by bypassing the membrane-mediated step and eliminates the need for accessory cells and regulatory signals (ie Interleukin-1). PMA stimulates protein kinase C activity while ionomycin increases intracellular calcium concentrations (Field et al, 1990). The depressed response to PMA + Iono further suggests that tumor-bearing significantly suppresses cell-mediated immunity in the host.

The increased response to both mitogens in the trained animals suggests an enhancement of cell-mediated immune function that could not be ascribed to changes in the relative composition of the splenic lymphocyte pool. Exercise training in animal models has been associated with both increased (Ferry et al, 1991) and decreased (Hoffman-Goetz et al, 1989 and Palhavani et al, 1988) proliferative responses to mitogens. Variable results presumably result from differences in the intensity and duration of exercise, sampling site and its timing with respect to the immunological testing. The physiological significance of these differences is not clear, particularly when data describing changes in the proportion of lymphocyte subsets is absent and changes in the size of the lymphocyte pool are evident.

Splenic natural killer cytotoxicity:

In the present experiment, a suppression of natural killer cell cytotoxicity was observed two weeks following tumor implantation. NK cells may play an important role in non-specific host defences against cancer and infectious disease (Herberman and Ortaldo, 1981). It is reasonable to assume that tumor development should be associated with or preceded by depressed natural immunity thereby impairing the ability of the host to reject the transformed cells. The increase in natural killer cell cytotoxicity three weeks following tumor implantation suggests an enhanced non-specific immune response. The effectiveness of NK cells in antitumor immunity in later stages of malignancy may be dependent on the concurrent stimulation of T-cells and macrophages which produce cytokines in response to the presence of the tumor.

No effect of exercise training on natural killer cell cytotoxicity was observed in this study. Increased NK cell activity in trained subjects has been postulated to confer better resistance to infection and neoplasia (Simpson and Hoffman-Goetz, 1990) possibly through frequent enhancement of cytotoxic mechanisms during and persisting after acute exercise. Acute exercise did not induce changes in natural killer cell activity in normal animals subject to the same exercise protocol (Chapter 3), therefore it was not surprising that the non-specific immune response in trained tumor-bearing animals was not different from that of their sedentary controls. The stress associated with CO_2 asphyxiation at the time of sacrifice was assumed to be the

same for all groups.

Mononuclear cell populations in spleen:

There was no difference in the relative percentage of any cell population measured, thus the effects of exercise training on mitogen response could not be attributed to changes in the relative proportion of different cell types in the spleen. Although it has been reported that training, apart from the immediate stress of acute exercise, augments the expression of T-cells in the spleen (Hoffman-Goetz et al, 1989), this effect was not apparent in the present study.

Overall, the results the present study demonstrate that dietary L-glutamine supplementation, but not exercise, reduces tumor growth in this animal model.

Table 4-1: Effect of tumor progression on whole body and tumor weights

Group	Initial weight (g)	Final weight (g)	Spleen weight (g)	Tumor weight (g)	Tumor weight as % final body weight
t=0 wks (5)	175.3 ± 4.4	200.9 ± 6.0	0.428 ± 0.010	not applicable	not applicable
t=2 wks (10)	182.4 ± 4.0	211.6 ± 6.8	0.438 ± 0.018	3.0 ± 0.4[a]	1.4 ± 0.1[a]
t=3 wks (10)	183.2 ± 2.5	225.8 ± 4.1	0.498 ± 0.018	13.9 ± 1.2[b]	6.1 ± 0.5[b]

Values are mean ± SE. The number of animals in each group is indicated in parentheses. Significant effects by one-way analysis of variance procedure; values without a common superscript within a column are significantly different, $p<0.001$.

Table 4-2:
Effect of tumor progression on amino acid concentrations in arterial plasma

Weeks after implantation	t = 0 weeks	t = 2 weeks	t = 3 weeks
Amino Acid		nmol/mL	
Alanine	426.5 ± 1.3 (5)*	408.4 ± 26.9 (9)	357.8 ± 15.9 (10)
Arginine	78.2 ± 3.5 (5)	81.6 ± 7.7 (9)	74.0 ± 4.9 (10)
Aspartate	15.8 ± 1.4 (5)[a]	22.1 ± 2.9 (8)[b]	14.5 ± 1.5 (9)[a]
Glutamate	200.6 ± 8.0 (5)	171.0 ± 19.9 (9)	144.9 ± 14.4 (9)
Glutamine	1108.5 ± 60.9 (5)	1007.4 ± 46.4 (9)	909.0 ± 60.8 (10)
Glycine	530.0 ± 54.7 (5)	507.4 ± 32.2 (9)	547.4 ± 21.6 (10)
Isoleucine	156.8 ± 7.0 (5)[a]	135.4 ± 7.7 (9)[a]	108.6 ± 8.0 (10)[b]
Leucine	256.6 ± 12.1 (5)[a]	218.2 ± 13.2 (9)[a]	174.1 ± 13.6 (10)[b]
Lysine	854.3 ± 17.8 (5)	814.7 ± 40.3 (9)	702.0 ± 36.4 (10)
Phenylalanine	70.1 ± 2.0 (5)	62.9 ± 2.3(9)	64.0 ± 2.6 (10)
Serine	454.0 ± 19.0 (5)	422.6 ± 23.8 (9)	407.6 ± 16.2 (10)
Threonine	400.3 ± 19.4 (5)	369.5 ± 22.5 (9)	333.8 ± 13.5 (10)
Tyrosine	120.6 ± 9.3 (5)[a]	106.7 ± 7.8 (9)[ab]	89.6 ± 7.2 (10)[b]
Valine	342.9 ± 15.4 (5)[a]	292.4 ± 14.6 (9)[b]	232.02 ± 16.4 (10)[c]

* Values are means ± SE (n). Significant effects by one-way analysis of variance procedure; values without a common superscript within a row are significantly different, $p<0.05$.

Table 4-3: Effect of tumor progression on proliferative response to mitogens, expressed as stimulation indices

Weeks after implantation	t = 0 weeks	t = 2 weeks	t = 3 weeks
48 hours			
Con A	4.79 ± 1.31^{a}	1.78 ± 0.42^{b}	0.19 ± 0.19^{b}
PMA + Iono	9.80 ± 2.79^{a}	11.50 ± 1.16^{b}	1.43 ± 1.12^{c}
72 hours			
Con A	31.94 ± 2.82^{a}	13.43 ± 1.40^{b}	13.18 ± 2.09^{b}
PMA + Iono	31.47 ± 1.34^{a}	11.86 ± 0.97^{b}	15.97 ± 1.68^{b}

Significant effects by one-way analysis of variance procedure; values within a row without a common superscript are significantly different, $p<0.001$.

Table 4-4: Effect of progressive tumor growth on glucose metabolism in splenocytes*

Product	$^{14}CO_2$		Lactate		Pyruvate		L/P ratio	
	– glutamine	+ glutamine	– glutamine	+ glutamine	– glutamine	+ glutamine	– glutamine	+ glutamine
	nmol/2h/10^6 cells							
t=0 wk	7.03 ± 0.47 (5)^a	4.07 ± 0.43 (5)*	29.35 ± 2.55 (5)	22.43 ± 1.41 (5)*	7.77 ± 0.68 (5)^a	7.71 ± 0.54 (5)	3.34 ± 0.14 (5)^a	2.99 ± 0.30 (5)
t=2 wk	4.59 ± 0.22 (5)^b	3.67 ± 0.20 (10)	25.07 ± 0.62 (5)	22.70 ± 2.42 (10)	5.87 ± 0.54 (5)^b	7.05 ± 0.47 (9)	4.40 ± 0.37 (5)^{ab}	3.39 ± 0.30 (9)
t=3 wk	4.03 ± 0.29 (9)^b	2.86 ± 0.18 (10)*	30.93 ± 3.19 (9)	26.33 ± 1.91 (10)	5.72 ± 0.46 (9)^b	7.62 ± 0.51 (10)*	5.50 ± 0.43 (9)^b	3.52 ± 0.27 (10)*

*Incubation media contained 4 mM [U-^{14}C] glucose ± 1 mM glutamine. Values with different superscripts within a column are significantly different ($p<0.01$) by one-way analysis of variance procedure. *For each metabolite, significantly different ($p<0.01$) from control (- glutamine) condition as analyzed by paired t-test.

Table 4-5: Effect of progressive tumor growth on glutamine metabolism in splenocytes*

Product	$^{14}CO_2$		[^{14}C]-Glutamate		[^{14}C]-Aspartate		Ammonia	
	– glucose	+ glucose	– glucose	+ glucose	– glucose	+ glucose	– glucose	+ glucose
	nmol/2h/10^6cells							
t=0 wk	10.91 ± 0.60 (5)^a	5.79 ± 0.26 (5)*	13.62 ± 0.65 (5)	8.62 ± 0.46 (5)*	8.62 ± 0.46 (5)	1.84 ± 0.17 (5)*	21.49 ± 2.42 (5)	37.79 ± 3.64 (5)^{a*}
t=2 wk	5.77 ± 1.24 (4)^b	4.26 ± 0.47 (10)	9.18 ± 0.57 (4)	8.23 ± 0.56 (9)	8.23 ± 0.56 (5)	1.26 ± 0.19 (8)*	14.07 ± 0.43 (3)	16.12 ± 2.19 (10)^b
t=3 wk	7.89 ± 0.73 (10)^b	4.47 ± 0.50 (10)*	12.50 ± 1.73 (9)	9.79 ± 1.03 (9)	8.89 ± 0.64 (9)	1.73 ± 0.12 (9)*	21.88 ± 1.94 (10)	26.34 ± 4.26 (10)^b

*Incubation media contained 1 mM [U-^{14}C] glutamine ± 4 mM glucose. Values with different superscripts within a column are significantly different ($p<0.01$) by one-way analysis of variance procedure. *For each metabolite, significantly different ($p<0.01$) from control (- glucose) condition as analyzed by paired t-test.

Table 4-6: Diet and exercise effects on whole body and tumor growth

Group	Initial weight (g)	Final weight (g)	Spleen weight (g)	Tumor weight (g)	Tumor weight as % final body weight
HGLN+ (10)	204.7 ± 3.5	215.8 ± 3.4	0.480 ± 0.011	4.9 ± 0.6	2.4 ± 0.3
SGLN+ (10)	206.3 ± 3.5	224.8 ± 3.4	0.513 ± 0.011	6.7 ± 0.6	3.0 ± 0.3
HGLN– (10)	208.6 ± 3.5	212.9 ± 3.4	0.452 ± 0.011	8.8 ± 0.6	4.3 ± 0.3
SGLN– (5)	210.0 ± 5.0	226.3 ± 4.8	0.496 ± 0.014	8.6 ± 0.7	4.5 ± 0.4
Significance*	NS	p<0.001 exercise	p<0.005 exercise	p<0.0001 diet	p<0.0001 diet

Values are means ± SE. The number of animals in each group is indicated in parentheses. *Significant effects by two-way analysis of variance procedure. Abbreviations used: H, exercise; S, sedentary; Gln+, glutamine supplemented diet; Gln-, non-glutamine supplemented diet.

Table 4-7: Amino acid concentrations in arterial plasma of tumor-bearing rats

Diet Group	Glutamine+		Glutamine-		Significance**	
Activity Level	Exercise (H)	Sedentary (S)	Exercise (H)	Sedentary (S)	Diet	Exercise
Amino Acid	nmol/mL					
Alanine	425.8 ± 25.1 (8)*	413.7 ± 22.5 (10)	432.7 ± 22.5 (10)	406.7 ± 31.8 (5)	NS	NS
Arginine	99.0 ± 9.7 (8)	102.6 ± 10.4 (10)	84.7 ± 8.7 (7)	98.1 ± 12.3 (5)	NS	NS
Aspartate	20.6 ± 2.3 (9)	26.2 ± 2.2 (10)	15.7 ± 2.2 (10)	22.0 ± 3.4 (4)	NS	0.05
Glutamate	173.6 ± 14.5 (9)	152.4 ± 13.8 (10)	123.4 ± 13.8 (10)	131.9 ± 19.5 (5)	NS	0.05
Glutamine	734.1 ± 24.5 (9)	691.8 ± 23.2 (10)	695.6 ± 23.2 (10)	694.3 ± 32.8 (5)	NS	NS
Glycine	172.7 ± 13.7 (9)	146.4 ± 12.3 (10)	660.3 ± 13.7 (9)	548.3 ± 24.7 (5)	0.001	NS
Isoleucine	113.2 ± 10.3 (9)	124.4 ± 9.3 (11)	104.9 ± 9.7 (10)	112.0 ± 13.8 (5)	NS	NS
Leucine	179.6 ± 8.9 (10)	196.8 ± 10.0 (8)	140.0 ± 8.5 (11)	163.0 ± 12.6 (5)	0.001	0.05
Lysine	643.8 ± 22.9 (8)	582.6 ± 22.9 (8)	644.5 ± 20.5 (10)	624.6 ± 32.4 (4)	NS	NS
Phenylalanine	70.6 ± 2.8 (9)	71.2 ± 2.5 (11)	62.7 ± 2.8 (9)	70.0 ± 3.7 (5)	NS	NS
Serine	272.4 ± 22.6 (9)	256.9 ± 21.4 (10)	509.6 ± 21.4 (10)	504.1 ± 30.3 (5)	0.001	NS
Threonine	334.3 ± 20.3 (9)	417.9 ± 19.3 (10)	280.9 ± 19.3 (10)	369.6 ± 27.3 (5)	0.05	0.001
Tyrosine	71.0 ± 5.1 (8)	74.2 ± 4.5 (10)	67.6 ± 4.5 (10)	59.5 ± 6.4 (5)	NS	NS
Valine	203.7 ± 9.8 (8)	239.3 ± 8.7 (10)	174.0 ± 8.7 (10)	191.0 ± 12.3 (5)	0.001	0.01

*Values are means ± SE (n). **Significant effects by two-way analysis of variance; no significant interaction was found for any amino acid tested.

Table 4-8: Diet and exercise effects on proliferative response to mitogens, expressed as stimulation indices

	Glutamine +		Glutamine -	
	High (H)	Sedentary (S)	High (H)	Sedentary (S)
48 hours				
Con A	0	0	0	0
PMA + Iono	3.30 ± 0.67†	1.70 ± 0.74	5.13 ± 0.67†	1.81 ± 0.67
72 hours				
Con A	12.31 ± 2.20*	9.67 ± 2.41	14.46 ± 2.20*	6.45 ± 2.20
PMA + Iono	14.09 ± 1.16 ⁺	6.94 ± 1.16	14.44 ± 1.27⁺	5.83 ± 1.16

Significant effect of exercise by two-way analysis of variance procedure: *$p<0.05$, † $p<0.005$, ⁺$p<0.0001$ (n=6 animals/group).

Table 4-9: Percentages of mononuclear cell subsets in the spleen of tumor-bearing rats*

	Glutamine +		Glutamine -	
	Exercise (H) (n=10)	Sedentary (S) (n=10)	Exercise (H) (n=10)	Sedentary (S) (n=5)
Subset				
CD3+	57.44 ± 2.78	57.90 ± 2.78	51.29 ± 2.93	58.63 ± 3.93
CD4+	39.02 ± 2.64	37.40 ± 2.64	33.99 ± 2.64	40.14 ± 3.74
CD8+	17.70 ± 1.13	18.63 ± 1.13	16.19 ± 1.19	18.57 ± 1.60
NK cells	8.40 ± 1.16	6.68 ± 1.16	6.84 ± 1.16	5.34 ± 1.16
CD20+	22.31 ± 1.99	22.07 ± 2.18	19.16 ± 0.79	18.38 ± 1.69
ϕ	8.86 ± 1.22	9.46 ± 1.22	6.54 ± 1.29	8.37 ± 1.63
CD4+/CD8+	2.27 ± 0.16	2.00 ± 0.16	2.06 ± 0.16	2.19 ± 0.23

Values are means ± SE (n). *NS, two-way analysis of variance procedure.
ϕ = macrophages

Figure 4-1: Effect of tumor progression on whole body weight gain

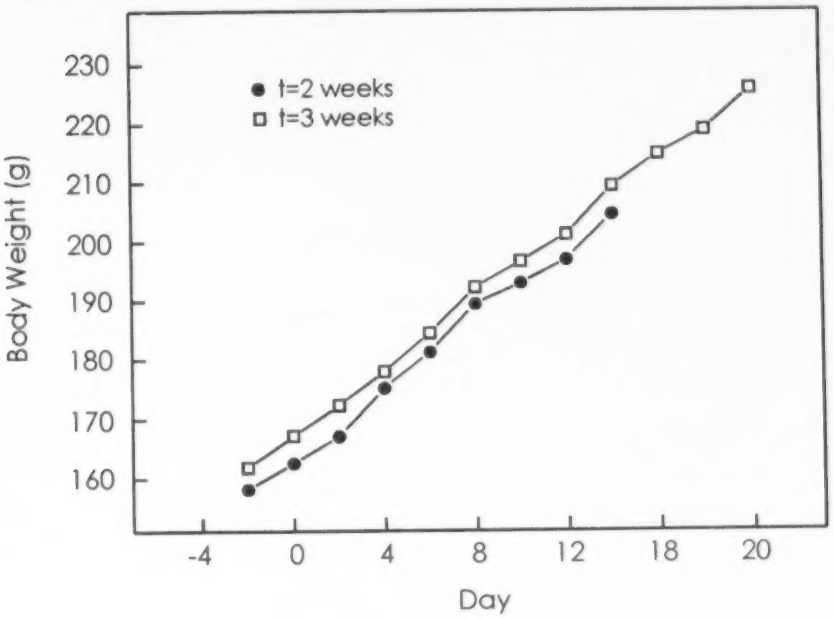


Figure 4-2: Effect of tumor progression on average daily food intake

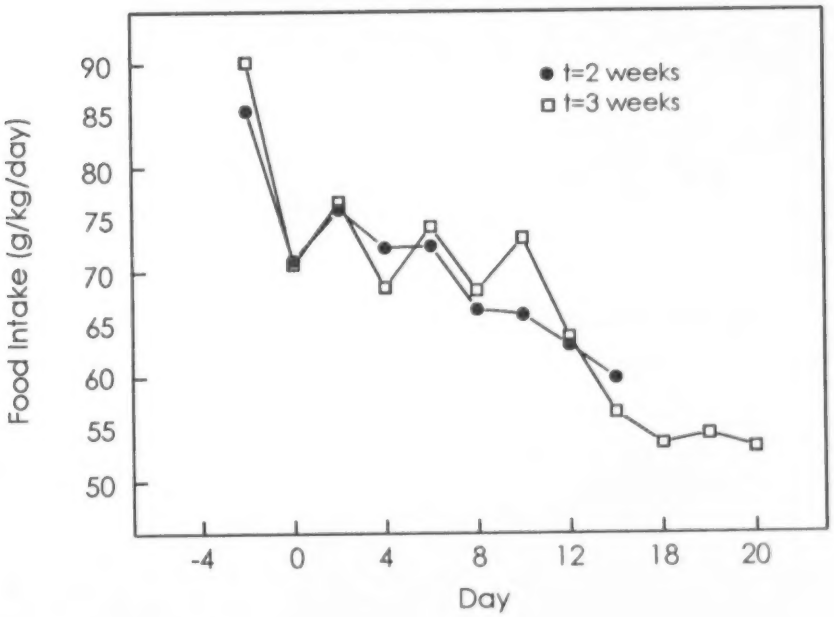
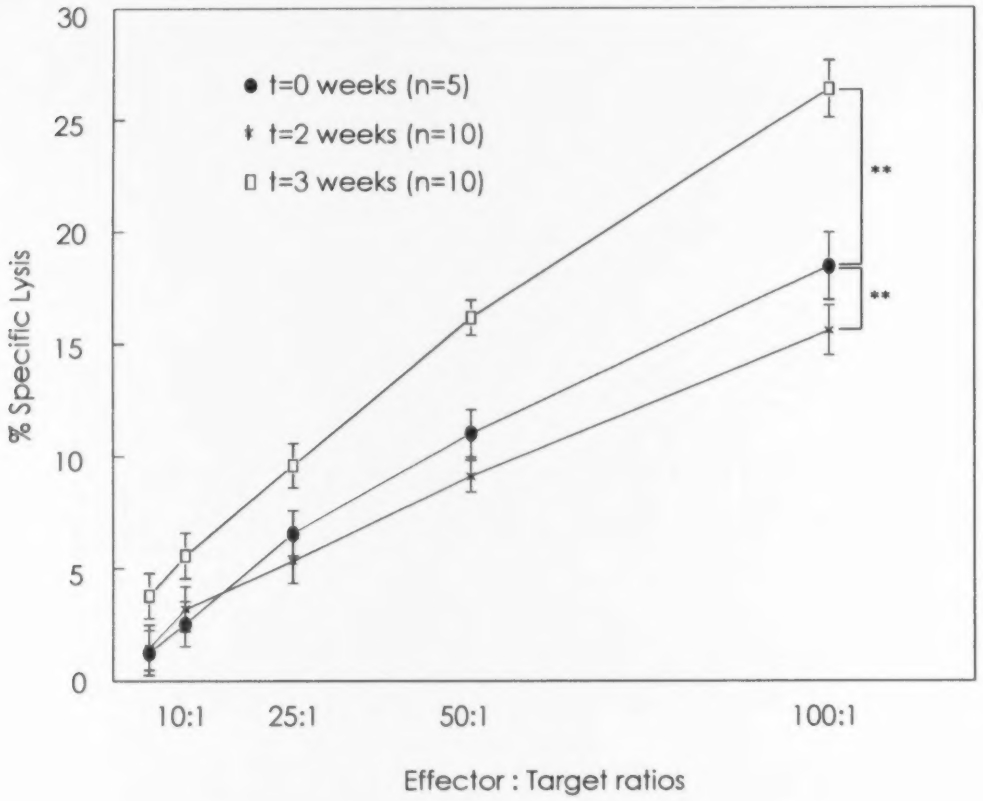
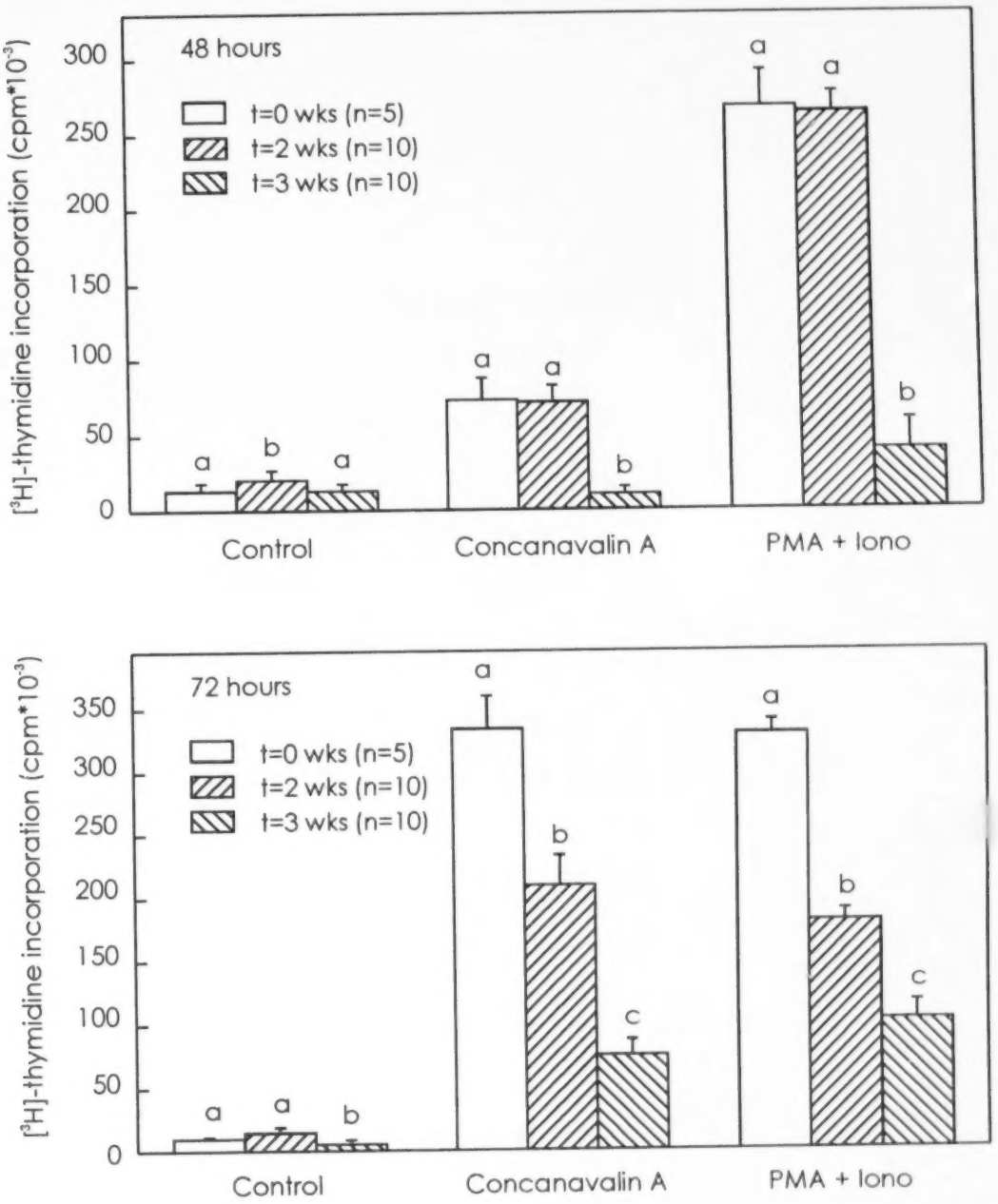


Figure 4-3: Effect of tumor progression on splenic natural killer cytolytic activity in female rats



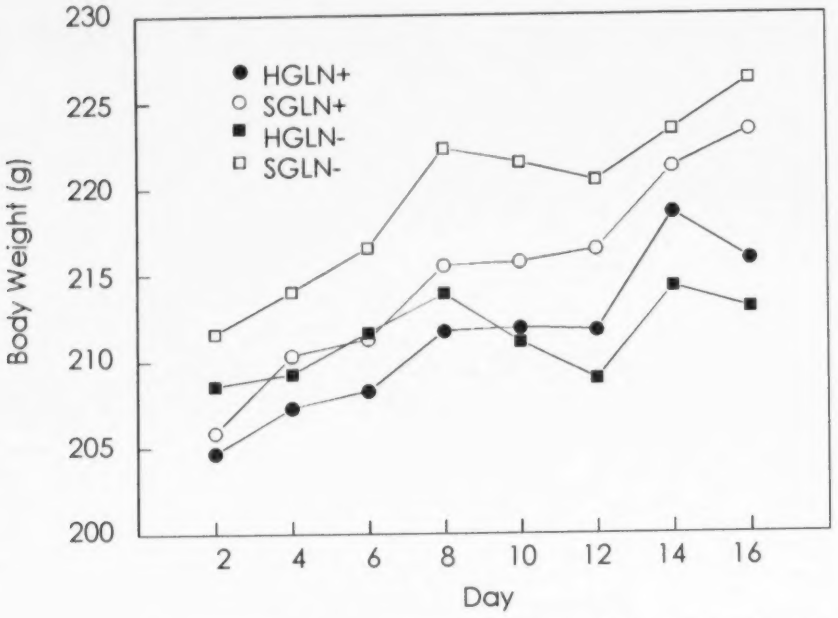
Values are means ± SE. **p<0.001, repeated measures analysis of variance procedure.

Figure 4-4: Effect of tumor progression on splenic response (mean ± SE) to Concanavalin A and PMA + Iono



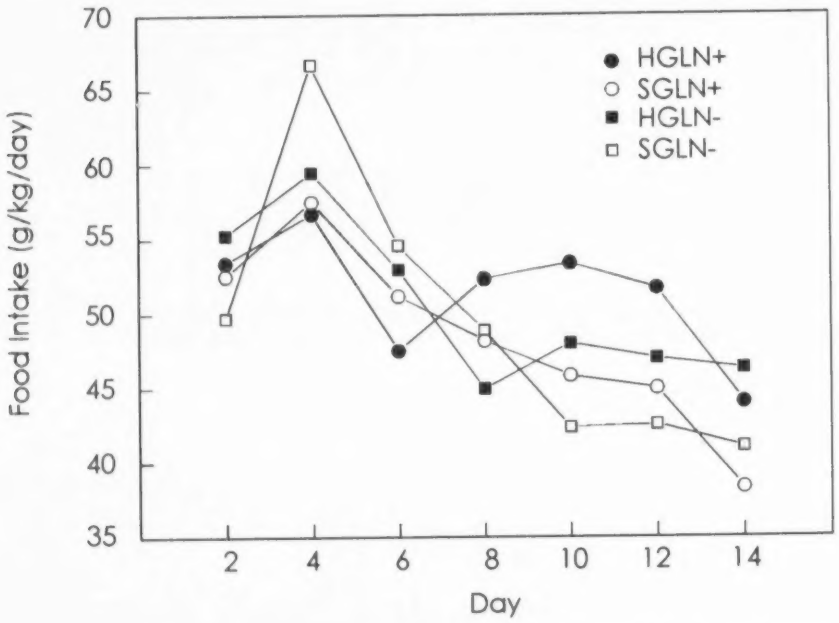
*Significant effects by one-way analysis of variance procedure.
Values without a common letter are significantly different, $p<0.05$.

Figure 4-5: Whole body weight gain in tumor-bearing animals



NS, two-way repeated measures analysis of variance.

Figure 4-6: Average daily food intake in tumor-bearing animals



NS, two-way repeated measures analysis of variance.

Figure 4-7: Effect of glutamine supplementation on arterial plasma amino acid concentrations in tumor-bearing rats

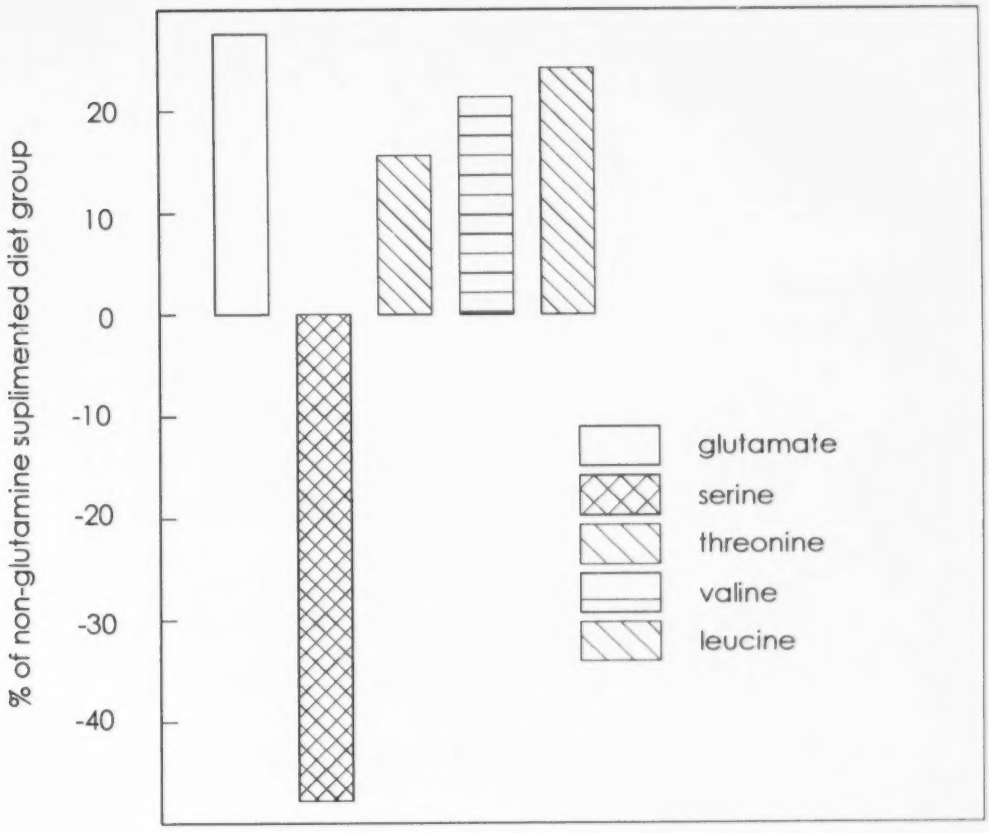


Figure 4-8: Effect of exercise on arterial plasma amino acid concentrations in tumor-bearing rats

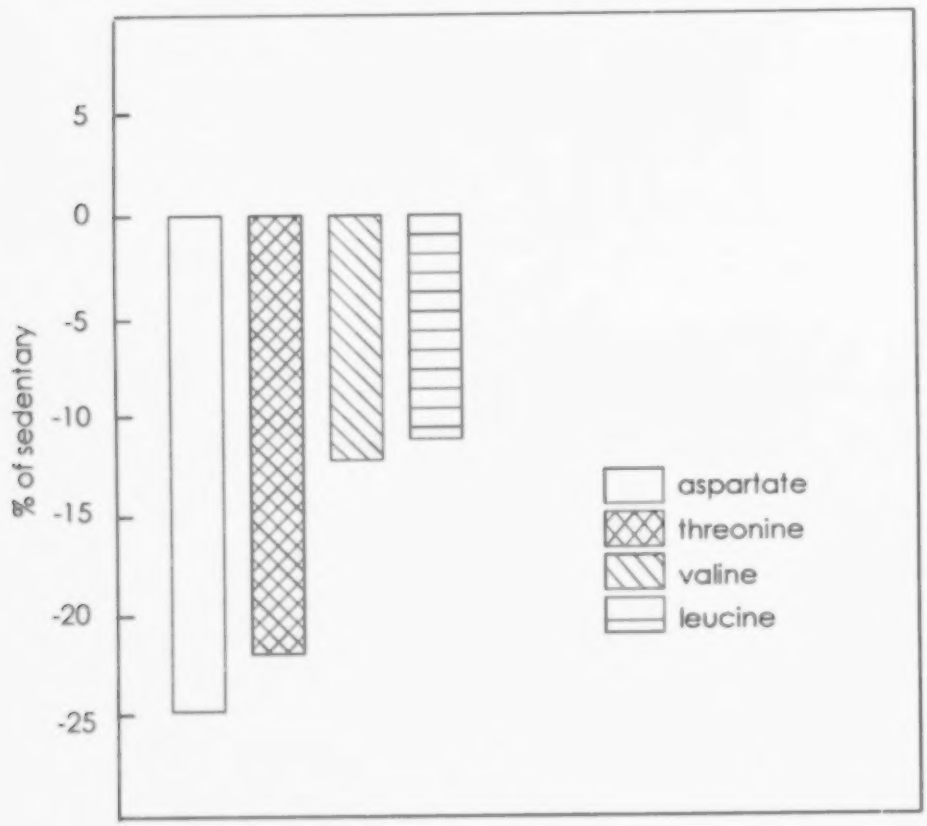
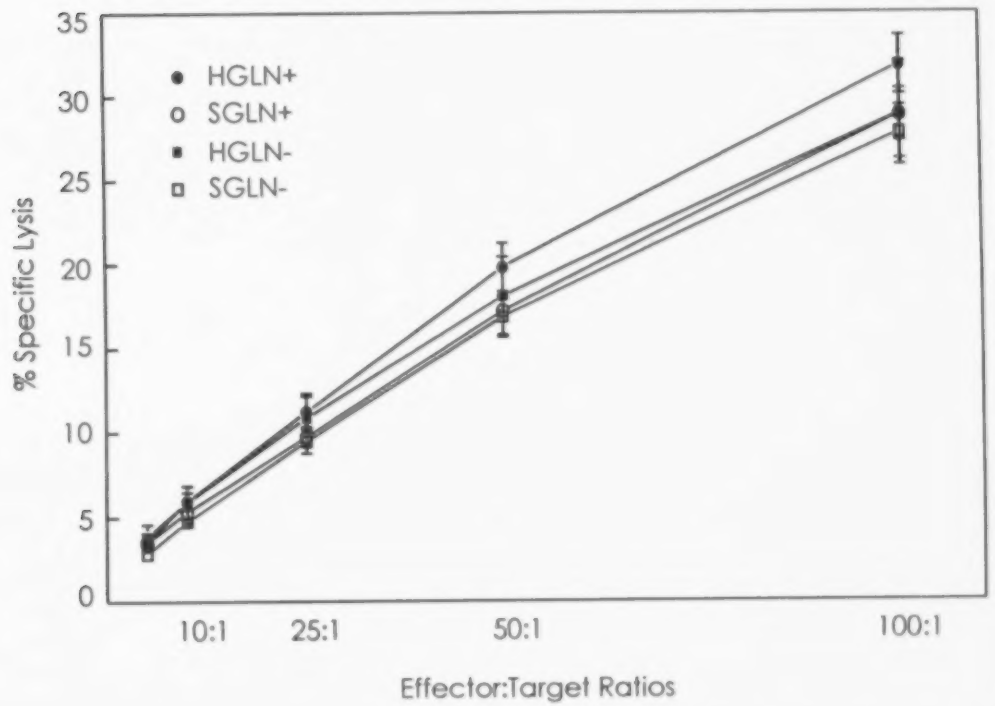
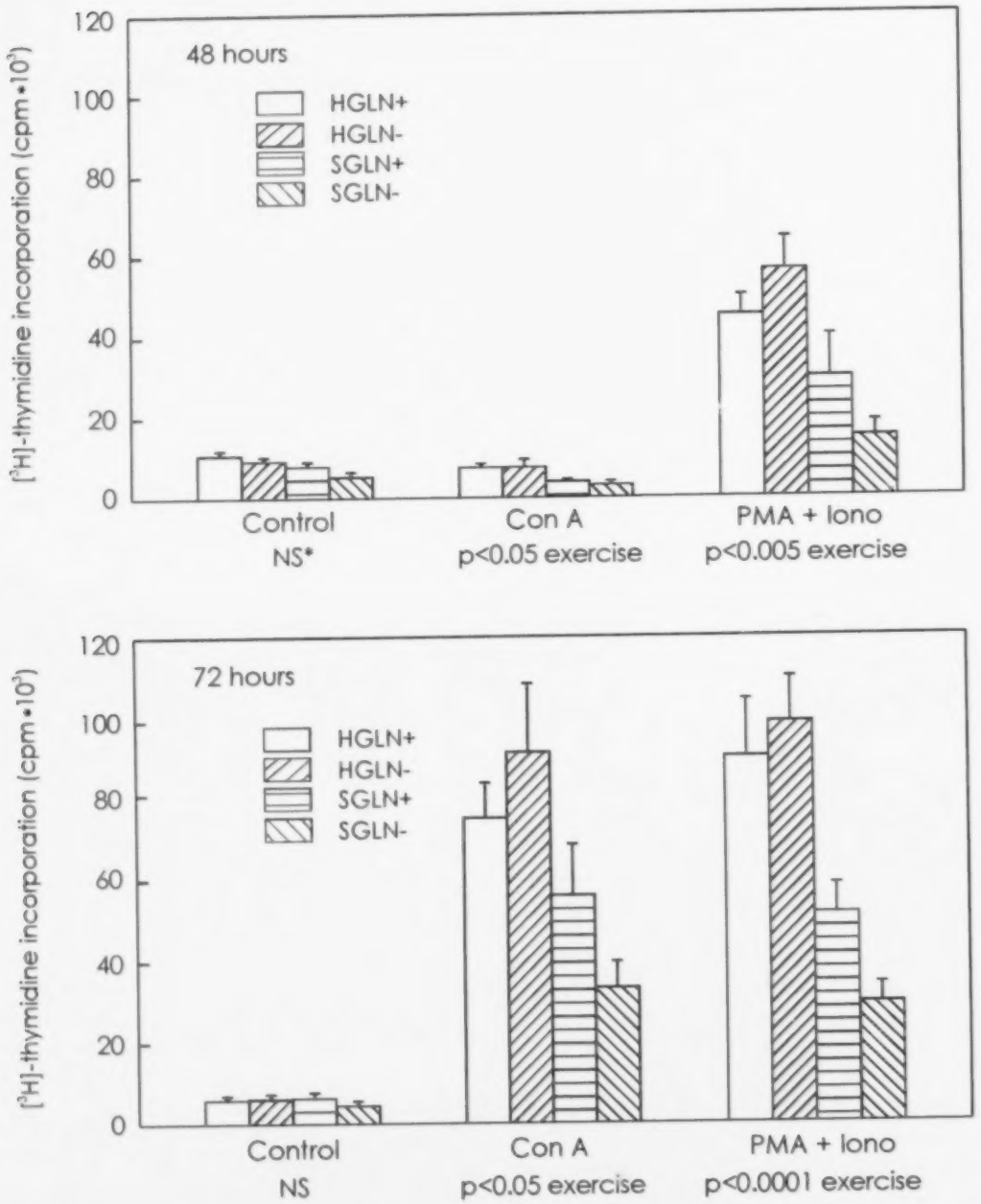


Figure 4-9: Effect of diet and exercise on splenic natural killer cytolytic activity in tumor-bearing rats



No significant effect of diet or exercise as analyzed by two-way repeated measures analysis of variance, n=6 animals/group.

Figure 4-10: Mitogen response (mean ± SE) of splenocytes to Con A or PMA + Iono in tumor-bearing rats



Significant effects by two-way analysis of variance procedure, n=6 animals/ group. *NS indicates no significant effect of diet or exercise.

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5. GLUCOSE AND GLUTAMINE METABOLISM IN FRESHLY ISOLATED MORRIS HEPATOMA 7777 CELLS

A. INTRODUCTION

An adequate supply of glutamine appears to be necessary for tumor growth since blockade of glutamine utilization leads to a dramatic decrease in the growth rate of experimental tumors *in vitro* and *in vivo* (Chance et al. 1988; Chance et al. 1990). The reduction in growth of transplantable rat hepatomas observed following treatment with the anti-glutamine metabolite, acivicin, appears to be directly related to the inhibition of enzymes necessary for the biosynthesis of purine and pyrimidine nucleotides from glutamine (Chance et al. 1988). The activities of these enzymes are elevated in rat hepatomas thus their inhibition is considered a desirable target for anti-neoplastic therapy. In a similar regard, glucose and glutamine are quantitatively important for energy provision in hepatoma cells. Detailed knowledge of the utilization and metabolic fate of these substrates and of the factors controlling their metabolism may be of considerable importance in understanding energy utilization in these cells. This information is also necessary to identify desirable targets for anti-neoplastic therapy, dietary or otherwise.

Isolated cell preparations are amenable to controlled studies of energy metabolism in hepatoma cells. They are free of interference from other tissues thus clarifying the quantitative significance of a single factor on flux through the energy yielding pathways. *In vitro*, an experimental condition can easily be manipulated to test a large number of biologically significant hypotheses and the use of several preparations allows direct comparison between control and treatment groups within the same group of cells. The purpose of the present study was to establish the relative importance of glucose and glutamine as energy substrates in freshly isolated Morris hepatoma 7777 cells.

B. MATERIALS AND METHODS

Tumor isolation:

Hepatoma cells were isolated by modification of the method described by Mares-Perlman and Shrago (1988). One gram of viable tissue was minced with scissors and suspended in 25 mL cold Ca^{++}/Mg^{++} free Hanks' Balanced Salt Solution (HBSS, pH 7.4) with bovine serum albumin (BSA, 5% w/v; Sigma Chemicals, St Louis, MO) and type

VII collagenase (200 units/mL; Sigma Chemicals, St Louis, MO). Flasks were gassed with 95% O_2-5% CO_2 and incubated with shaking for 15 minutes. The tissue suspension was filtered through 250 µm nylon mesh, resuspended in 15 mL of fresh media, gassed and incubated for an additional 60 minutes. The filtrate from the first incubation was discarded. The suspension from the second incubation was filtered through a syringe tip filter (100 µm) and centrifuged at 200 g for 10 minutes. The pellet was resuspended in HBSS + 0.5% BSA, layered on a density gradient (Histopaque; Sigma Chemicals, St Louis, MO) and centrifuged at 400 g for 30 minutes. The cell interface was removed, washed three times in HBSS + 0.5% BSA and resuspended at a final concentration of $5x10^6$ cells/mL. Cell viability was determined by trypan blue exclusion and was always greater than 90%.

Tumor cell metabolism:

Triplicate incubations were performed at 37°C in 10 mL silicone treated tubes (Vacutainer, Becton Dickson). Freshly isolated tumor cells were incubated for two hours at a density of $0.5x10^6$ cells/mL as previously described for splenocytes (Wu et al, 1991). Cell concentration and time trials were previously performed to determine optimum cellular density and length of incubation (Figures A-2, A-3). The incubation media contained [U-^{14}C]-glutamine (1 mM, 0.15 µCi/µmol; NEN, Wilmington, DE) ± 4 mM glucose, [U-^{14}C]-glucose (4 mM, 0.15 µCi/µmol; NEN, Wilmington, DE) ± 1 mM glutamine or RPMI 1640 media (Fisher Scientific, Edmonton, AB) + 1 mM glutamine. Parallel incubations in which the media was acidified with 100 µL of 1.5 M perchloric acid before the addition of cells were run as blanks.

$^{14}CO_2$ was measured in a Beckman 5000 liquid scintillation counter using modified Bray's solution (Bray, 1960). Ammonia (Bergmeyer and Beutler, 1985), lactate (Hohorst, 1974) and pyruvate (Czok and Lamprecht, 1974) concentrations in the media were determined by spectrophotometric enzymatic methods. [^{14}C]-glutamate was separated by ion exchange chromatography (Wu et al, 1991) and measured in a Beckman 5000 liquid scintillation counter using Scintiverse cocktail (Fisher Scientific, Edmonton, AB).

Soluble endproducts of glucose and glutamine metabolism (glutamate, aspartate, ammonia, lactate and pyruvate) are measured immediately following cell lysis and thus reflect both intracellular concentrations and the release of product into the media.

Statistical analysis:

The effects of diet treatment and exercise were determined by two-way analysis of variance procedure. All data is expressed as mean ± standard error (SE).

C. RESULTS

Glucose metabolism - Lactate was the major measured endproduct of glucose metabolism by tumor cells (Table 5-1). More glucose was metabolized to lactate than was oxidized to CO_2. The addition of glutamine to the media did not alter CO_2 or lactate production. Inasmuch as pyruvate production decreased ($p<0.01$) in the presence of glutamine, the lactate/pyruvate ratio in the media increased ($p<0.01$). Neither diet nor exercise had any significant effect on the metabolic endproducts measured.

Glutamine metabolism - Glutamate and ammonia were the major measured endproducts of glutamine metabolism by tumor cells (Table 5-2). Similar to glucose, fewer glutamine carbons were recovered by complete oxidation to CO_2 than were recovered in glutamate. Aspartate was not detectable in the incubation media of these cells. The addition of glucose to the media significantly ($p<0.01$) decreased the production of CO_2 from glutamine but did not alter the production of other metabolites. No significant effect of diet or exercise was observed.

Incubation in RPMI media - Cells incubated in RPMI 1640 + 1 mM glutamine utilized aspartate, asparagine, serine, glutamine, histidine, threonine, arginine, tyrosine, tryptophan, methionine, valine, phenylalanine, leucine and isoleucine and produced glutamate, glycine and alanine (Figure 5-1). Of the amino acids present in the media, glutamine was utilized to the greatest extent.

D. DISCUSSION

Glucose and glutamine metabolism in Morris hepatoma 7777 cells:

There is a lack of information about the relative importance of glutamine and glucose for energy provision in tumor cells (Board et al, 1990). In the present study, the addition of glucose to the incubation media significantly ($p<0.01$) decreased the production of carbon dioxide from glutamine but did not alter the production of other metabolites. The results suggest that energy derived from glutamine can, at least partially, be replaced by glucose. In other studies of glucose and glutamine metabolism in malignant cells, glutamine utilization increased (Segura et al, 1989).

decreased (Medina et al, 1988) or did not change when glucose was added to the media. Glucose did not modify glutaminolytic flux yet glutamine changed the rate of glucose consumption in perifused Erlich ascites cells (Luque et al, 1990). The discrepancy in results can be attributed to differences in substrate concentration, cellular density, measurement technique, cell origin or length of incubation. The mechanism by which glutamine inhibits glycolysis has not been clarified, however, the inhibition of acetyl coA synthesis and pyruvate oxidation has only a small effect (Matsuno, 1987).

Tumors metabolize glucose via glycolysis at high rates even in the presence of normal oxygen availability (Argiles and Azcon-Bieto, 1988). As a result of the high glycolytic rate, large quantities of pyruvate are generated together with an increase in the cytosolic NADH/NAD+ ratio which favors the reduction of pyruvate to lactate through the action of lactate dehydrogenase (Argiles and Azcon-Bieto, 1988). The results of most studies support the view that it is not enzyme capacity that is responsible for the low rates of oxidation but that metabolic control of enzyme activity may be responsible (Board et al, 1990). Knowledge of this mechanism may be of considerable importance in understanding energy formation in these cells and in identifying suitable targets of antineoplastic therapy.

Pyruvate production from glucose in the presence of glutamine decreased in the present study. The complete pathway of glucose utilization in these cells has not been characterized and may reflect the diversion of pyruvate into other metabolic pathways. The intracellular lactate-to-pyruvate (L/P) ratio is often used to indicate the oxidation-reduction state of cytoplasmic pyridine nucleotides. In the present study, the L/P ratio was determined in the media immediately after cell lysis and thus the increased redox state represents the balance between release into the media and reutilization. The extent to which lactate and pyruvate are reutilized in these cells is not known.

In the presence of both energy substrates, the rate of glutamine oxidation was more than 20x greater in Morris hepatoma cells than in splenocytes incubated under identical conditions (Figure 5-2). If the relative rates of consumption *in vitro* could reasonably be extrapolated to the tumor-bearing host, the competitive demand for limited quantities of glucose and glutamine in plasma could have great implications on host immunocompetence. The severity of this effect would depend on the ability of both cell types to derive energy from other substrates. Arteriovenous differences

for acetoacetate, β-hydroxybutyrate, glucose, lactate, glutamine and other amino acids have been measured across the Morris hepatoma 7777 *in vivo* in fasted rats (Sauer and Dauchy, 1983). Glutamine was utilized most rapidly and the rates of glucose and glutamine utilization appeared to be directly dependent on the rate of supply. Ammonia was released into venous blood in large quantities. In freshly isolated Morris hepatoma 7777 cells, glucose and glutamine were quantitatively more important energy substrates than palmitate or β-hydroxybutyrate (Mares-Perlman and Shrago, 1988). The high rates of glutamine utilization and ammonia production are in agreement with those described herein.

Incubation in RPMI media:

The pattern of amino acid metabolism in cells incubated in RPMI media in this study (Figure 5-1) closely resembles that reported for perifusion studies in Erlich ascites tumors (Marquez et al, 1989). The results suggest high rates of glutamine utilization even when the glucose concentration in RPMI (10 mM) exceeds normal fasting and fed values. Because of extensive patterns of amino acid reutilization and interconversion, rates of utilization for individual amino acids in complete media may not represent absolute rates of synthesis or utilization by tumor cells.

Amino acids are utilized in tumor cells for oxidation and protein synthesis. They are also C and N donors for the synthesis of pyrimidine and purine nucleotides essential for tumor proliferation. Carefully designed studies of amino acid utilization by malignant tissues will provide insight into the important ramifications of cancer on whole body amino acid utilization and protein turnover. A method for growing transplantable tumors in rats which restricts the tumor blood supply and drainage to a single artery and vein was described by Sauer and Dauchy (1983). The tumor preparations were used to measure arteriovenous differences for whole-blood amino acids, ammonia, glucose and lactic acid *in vivo*. Since individual tumors of the same strain can show different metabolic responses to apparently identical *in vivo* conditions (Sauer et al, 1982), detailed analyses of C and N balance across single tumors using a method such as that described by Sauer and Dauchy (1983) will be required to precisely define patterns of amino acid utilization.

Overall, this study demonstrates that although the provision of glucose can modify glutamine oxidation in freshly isolated Morris hepatoma 7777 cells, the consumption of glutamine by these cells is still very high. The results also demonstrate a diverse pattern of amino acid utilization by tumor cells, even in the presence of high glucose concentrations.

Table 5-1: Endproducts of glucose metabolism in freshly isolated Morris hepatoma 7777 cells*

Product	$^{14}CO_2$		Lactate		Pyruvate		L/P ratio	
	– glutamine	+ glutamine	– glutamine	+ glutamine	– glutamine	+ glutamine	– glutamine	+ glutamine
Group	nmol/2h/0.5×10^{6}cells							
HGLN+	19.97 ± 2.14 (9)	19.12 ± 3.03 (9)	68.06 ± 2.79 (10)	69.38 ± 8.69 (10)	20.61 ± 1.10 (10)	12.38 ± 0.76 (10)*	3.26 ± 0.10 (10)	5.71 ± 0.53 (10)*
SGLN+	22.96 ± 2.14 (9)	18.85 ± 3.03 (9)	61.04 ± 2.79 (10)	63.67 ± 4.37 (10)	20.84 ± 1.10 (10)	12.49 ± 0.76 (10)*	2.95 ± 0.10 (10)	5.82 ± 0.56 (9)*
HGLN–	22.46 ± 2.27 (8)	18.86 ± 3.03 (9)	60.60 ± 2.79 (10)	59.43 ± 4.37 (10)	21.77 ± 1.10 (10)	11.91 ± 0.76 (10)*	2.86 ± 0.10 (10)	5.25 ± 0.53 (10)*
SGLN–	21.66 ± 2.88 (5)	21.58 ± 4.07 (5)	64.99 ± 3.94 (5)	69.54 ± 3.46 (16)	22.36 ± 1.56 (5)	12.38 ± 1.08 (5)*	2.94 ± 0.14 (5)	5.49 ± 0.75 (5)*

Values are means ± SE (n). *Incubation media contained 4 mM [U-^{14}C] glucose ± 1 mM glutamine. No significant effect of diet or exercise on any metabolic endproduct measured as analyzed by two-way analysis of variance procedure. *Significantly different ($p<0.01$) from control (- glutamine) condition as analyzed by paired t-test. Abbreviations used: H, exercise; S, sedentary; Gln+, glutamine supplemented diet; Gln-, non-glutamine supplemented diet.

Table 5-2: Endproducts of glutamine metabolism in freshly isolated Morris hepatoma 7777 cells*

Product	$^{14}CO_2$		[^{14}C]-Glutamate		Ammonia	
	– glucose	+ glucose	– glucose	+ glucose	– glucose	+ glucose
Group	nmol/2h/0.5×10^{6}cells					
HGLN+	65.91 ± 5.63 (10)	42.81 ± 3.62 (8)*	34.19 ± 1.79 (10)	39.61 ± 3.54 (10)	54.67 ± 11.61 (10)	61.77 ± 8.69 (9)
SGLN+	69.99 ± 5.63 (10)	43.52 ± 3.62 (8)*	35.87 ± 1.79 (10)	42.54 ± 3.54 (10)	60.65 ± 12.98(8)	82.73 ± 9.21 (8)
HGLN–	75.71 ± 5.63 (10)	47.39 ± 3.62 (8)*	34.44 ± 1.79 (10)	40.09 ± 3.54 (10)	65.11 ± 11.61 (10)	63.71 ± 8.69 (9)
SGLN–	70.63 ± 7.96(10)	48.39 ± 3.23 (10)*	37.00 ± 2.54 (5)	37.81 ± 5.01 (5)	73.02 ± 16.42 (5)	54.42 ± 11.65 (5)

Values are means ± SE (n). *Incubation media contained 1 mM [U-^{14}C] glutamine ± 4 mM glucose. No significant effect of exercise or diet on any metabolic endproduct measured as analyzed by two-way analysis of variance procedure. *Significantly different ($p<0.01$) from control (- glucose) condition as analyzed by paired t-test. No [^{14}C]-aspartate was detectable in the media.

Figure 5-1: Amino acid utilization by Morris hepatoma 7777 cells incubated in RMPI 1640 media + 1 mM glutamine (n=10 animals)

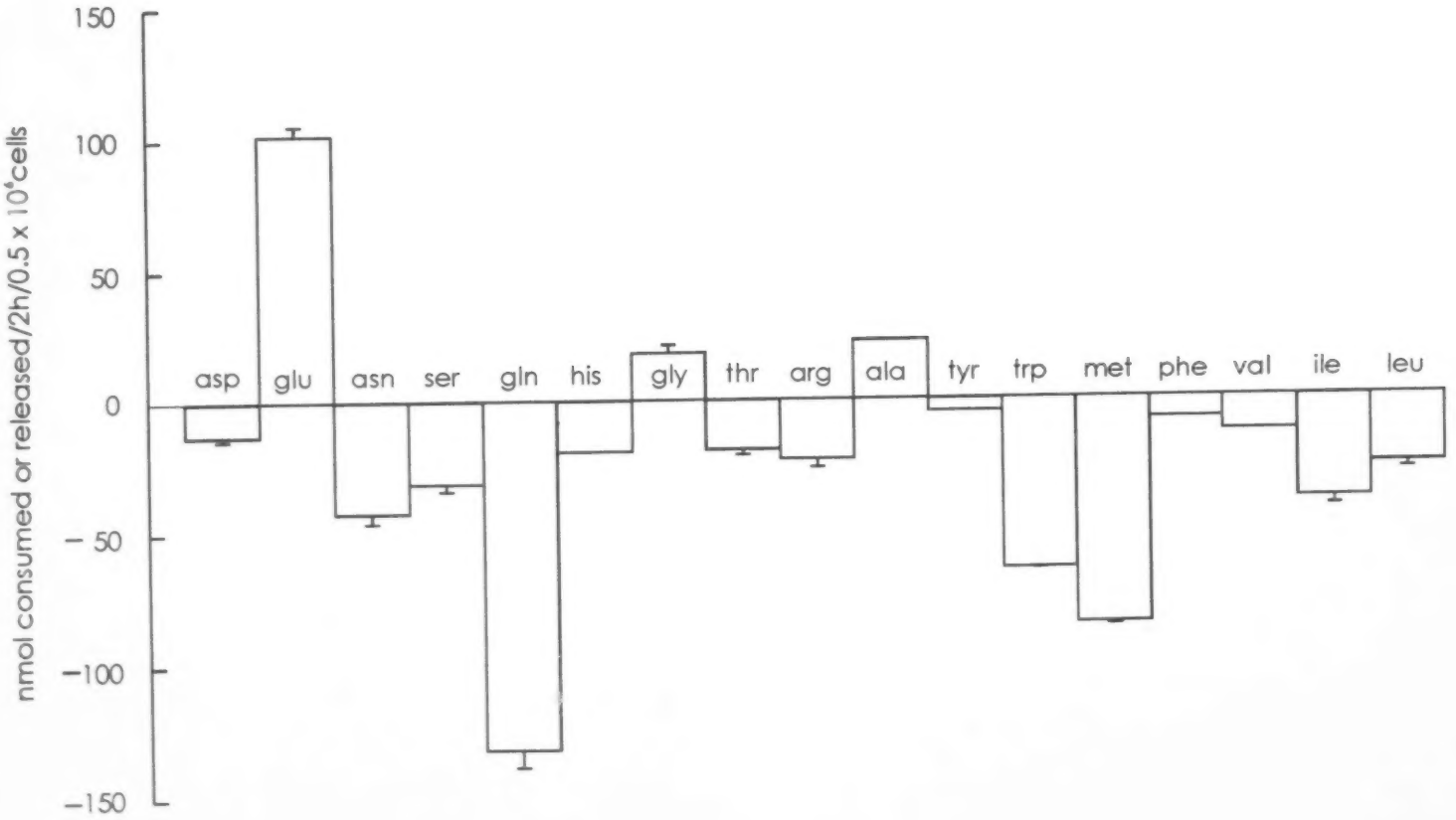
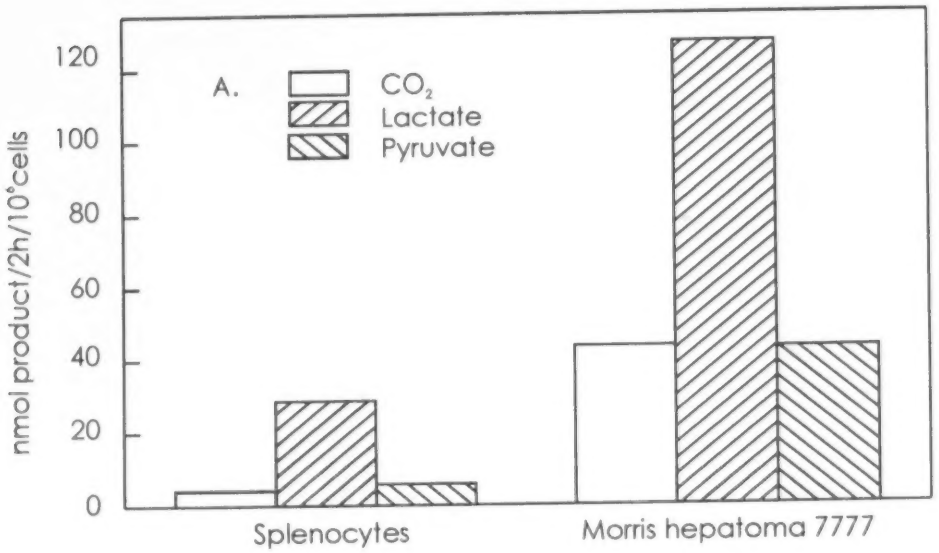
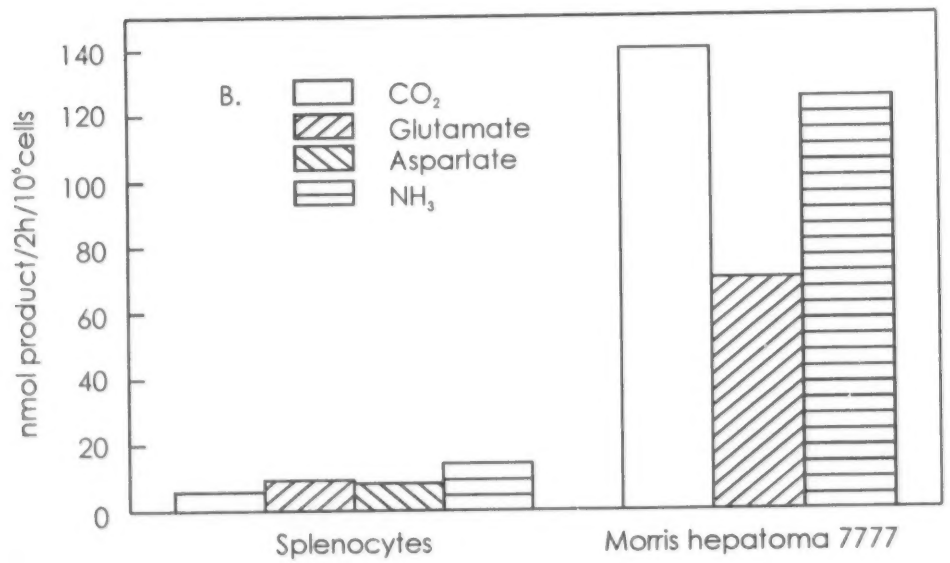


Figure 5-2: Relative rates of glucose (A,C) and glutamine (B,D) utilization in tissues of the tumor-bearing rat

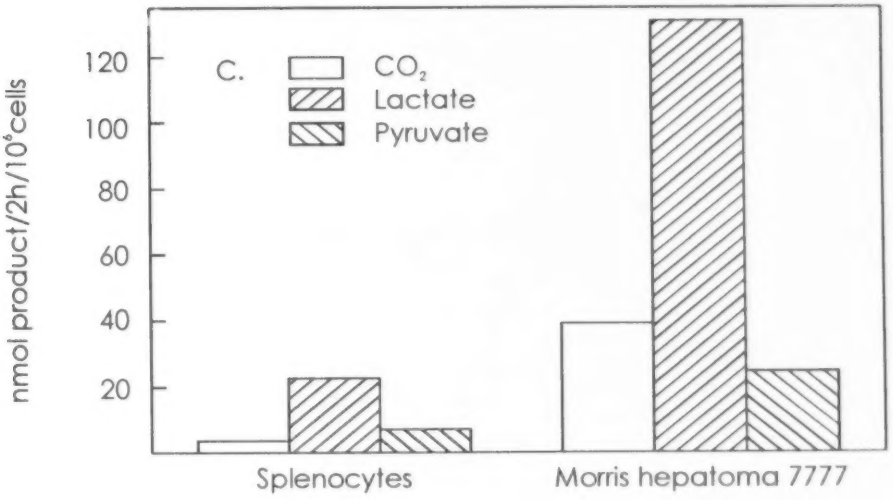


Incubation media contained 4 mM [U-^{14}C] glucose.

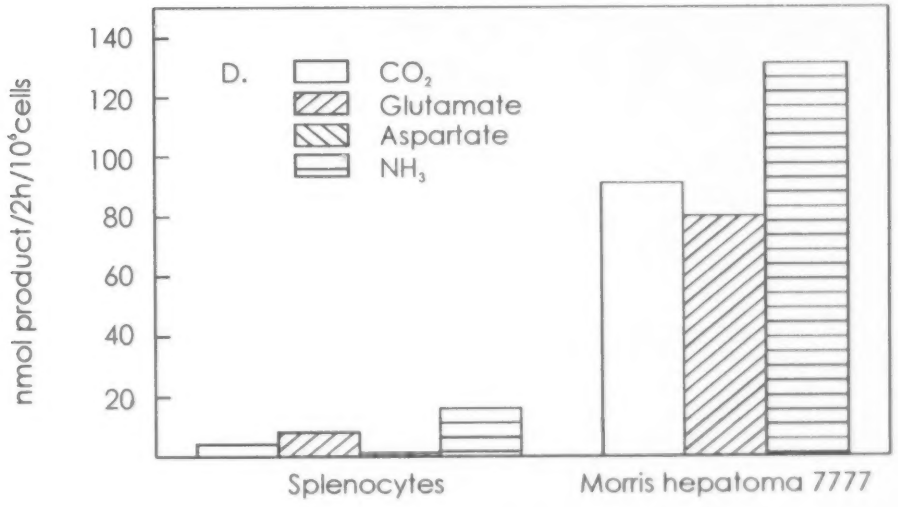


Incubation media contained 1 mM [U-^{14}C] glutamine. No aspartate was detectable in the incubation media of Morris hepatoma 7777 cells.

Figure 5-2: Relative rates of glucose (A,C) and glutamine (B,D) utilization in tissues of the tumor-bearing rat continued...



Incubation media contained 4 mM [U-^{14}C] glucose and 1 mM glutamine.



Incubation media contained 1 mM [U-^{14}C] glutamine and 4 mM glucose. No aspartate was detectable in the incubation media of Morris hepatoma 7777 cells.

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6. SUMMARY AND DISCUSSION

The objectives of this thesis were:

1. to characterize the effects of exercise on lymphocyte metabolism and function;
2. to establish the requirement for glutamine by immune cells during or following exercise;
3. to determine if dietary L-glutamine supplementation alters changes that occur in muscle protein metabolism and immune function during exercise;
4. to evaluate the temporal relationship between tumor development and immune responsiveness;
5. to examine concurrently the effects of dietary L-glutamine supplementation and exercise on specific parameters of immune function and experimental tumor development;
6. to assess the relative importance of glucose and glutamine as energy substrates in freshly isolated Morris hepatoma 7777 cells;

The objectives of this thesis were fulfilled as follows:

OBJECTIVE ONE: Acute exercise of moderate intensity did not modify glucose or glutamine metabolism in splenocytes.

OBJECTIVE TWO: Inasmuch as *in vitro* energy metabolism in splenocytes and plasma glutamine concentrations were unchanged immediately following exercise, no additional requirement for glutamine by immune cells was evident.

OBJECTIVE THREE: Net protein degradation in isolated epitrochlearis muscle and natural killer cytotoxicity in splenocytes were not different between trained animals and sedentary controls. Furthermore, neither parameter was altered by dietary L-glutamine supplementation.

OBJECTIVE FOUR: An apparent inverse relationship exists between tumor size and immune cell response to mitogens in tumor-bearing animals. The proliferative response to Con A and PMA + Iono was lowest in splenocytes isolated from animals with large tumor burdens.

OBJECTIVE FIVE: Dietary L-glutamine supplementation significantly reduced the growth of the Morris hepatoma 7777, when expressed both as absolute tumor weight

and tumor weight as a percentage of final body weight. Dietary manipulation did not result in altered mitogen response or natural killer cell activity in splenocytes, however. Inasmuch as measurement occurred 24 hours following the last bout of exercise, training induced an enhanced proliferative response to both Con A and PMA + Iono but did not alter splenic NK activity. Exercise did not alter tumor growth in this animal model.

OBJECTIVE SIX: The addition of glucose to the incubation media modified glutamine oxidation in freshly isolated Morris hepatoma 7777 cells. The consumption of both energy substrates by these cells, compared to immune cells, is very high.

The data presented in this thesis supports the previous suggestion that glutamine-enriched oral diets can support host glutamine metabolism without stimulating tumor growth (Klimberg et al, 1990). Indeed, the results indicate that dietary L-glutamine supplementation can be effective in reducing growth of the Morris hepatoma 7777. The results also document the changes in cell-mediated immune function associated with physical activity in the tumor-bearing host.

In normal animals, moderately intense exercise was not associated with changes in plasma glutamine concentration, non-specific immune responses measured *in vitro* or net protein degradation in isolated muscle. The results do not support an increased requirement for glutamine by immune cells during or following exercise and suggest that glutamine supplementation is not warranted in healthy individuals. Clearly, the ability of dietary glutamine to increase plasma and/or intramuscular glutamine concentrations depends on the extent of glutamine utilization by cells of the intestinal mucosa. Carefully designed exercise protocols of defined intensity and duration are needed to precisely identify the threshold workrate below which exercise protects the immune system and above which it becomes detrimental. It is also necessary to determine whether transient fluctuations in plasma glutamine concentrations that may occur during exercise are detrimental to the immune system.

Animal studies that have previously suggested that chronic exercise leads to a variety of immunological adaptations of humoral and cellular immunity have failed to describe the clinical implications of these adaptations, particularly as they relate to resistance against neoplasia. In tumor-bearing animals, progressive tumor growth was associated with a suppression of cell-mediated and non-specific immune responses and a decrease in plasma branch-chain amino acid concentrations.

Exercise did not significantly inhibit tumor growth but did improve splenic responsiveness to mitogen stimulation. It has been suggested that the enhancement of cytotoxic mechanisms during and after acute exercise are of benefit in preventing neoplasia. In the present study, no immediate or prolonged effect of exercise on natural killer cytotoxicity in either healthy or tumor-bearing animals was observed.

The mechanism by which dietary L-glutamine supplementation inhibited tumor growth is not clear but may be related to changes in substrate availability. Isolated hepatoma cells demonstrate high rates of non-oxidative glucose and glutamine metabolism and the potential to compete with host tissues for these substrates. Although dietary glutamine did not alter glutamine concentrations in plasma (0.70 mM), the precursors of glutamine synthesis, glutamate, leucine and valine, increased. It is reasonable to assume that by satisfying the demand for glutamine by cells of the intestinal mucosa in the tumor-bearing animal, endogenous glutamine is available for use by other tissues, including activated cells of the immune system. In the present study, the presence of the tumor was associated with increased [^{3}H]-thymidine incorporation in unstimulated splenocytes. This suggests that two weeks following implantation, the tumor increased the activity of immune system *in vitro*, thereby increasing its demand for glutamine. Attempts to clarify the precise mechanisms underlying these effects should be the focus of continuing investigation in this area.

Overall, it is apparent that dietary modulation can be combined with moderate amounts of physical activity to improve host defence mechanisms against neoplastic growth. The results of glutamine supplementation are promising in this animal model with a conveniently placed transplantable tumor and well defined diet and exercise regimes. The relevance of the present findings to other tumor models in other host species remains to be clarified, however. Further research is necessary to determine the effects of glutamine supplementation and exercise in the human population where environmental and genetic factors are difficult or impossible to control and tumors generally arise spontaneously.

A. *BIBLIOGRAPHY*

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APPENDIX

Table A-1: Diet Formulation

Ingredient	Gln +	Gln -
	g ingredient/100 g diet	
Casein (high protein)	25.65	25.65
Starch	21.00	19.00
Glucose (dextrose)	19.75	19.75
Non-nutritive cellulose	4.75	4.75
Vitamin Mix, AOAC	0.95	0.95
Mineral Mix, Bernhart-Tomarelli	4.80	4.80
Choline	0.25	0.25
Inositol	0.60	0.60
L-methionine	0.25	0.25
L-glutamine	2.00	0.00
L-glycine	0.00	4.00
Linseed Oil	0.40	0.40
Beef Tallow	8.00	8.00
Safflower Oil	11.60	11.60
Total	100.00	100.00
P/S ratio*	0.94	0.94

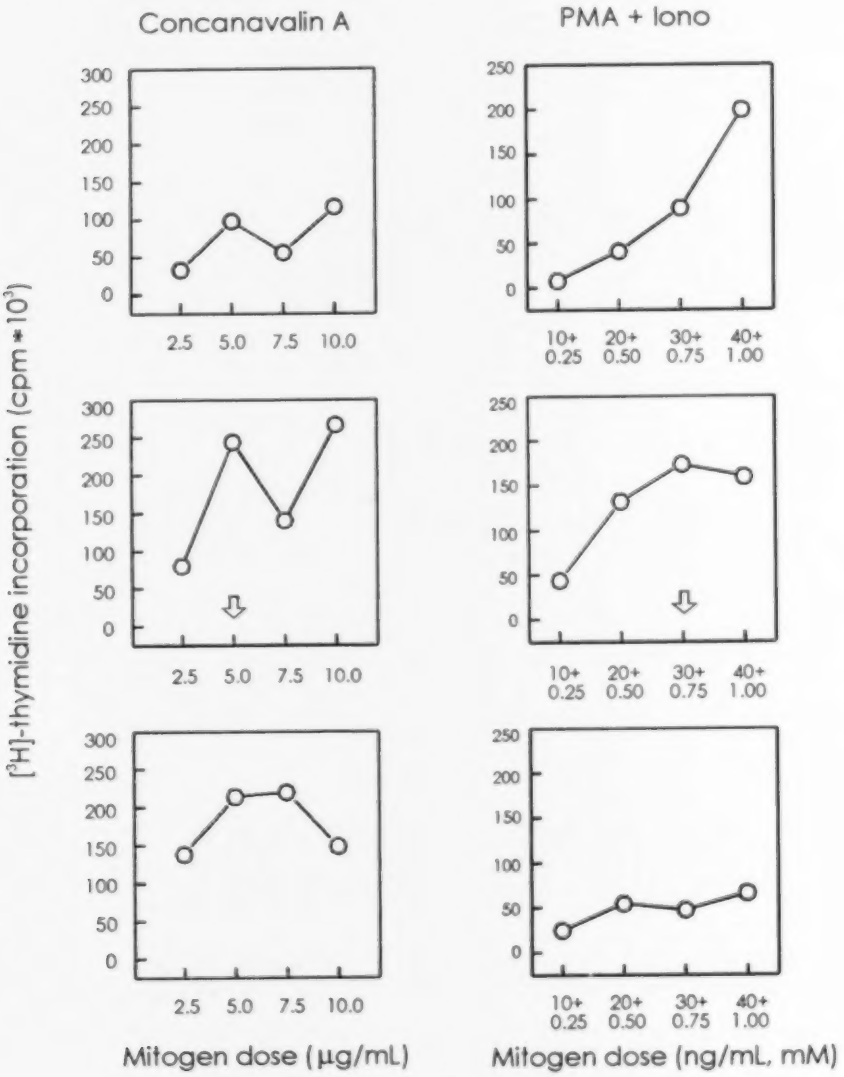
*As determined by gas liquid chromatography

Table A-2:
Amino Acid Composition of Casein*

	(g/100 g ingredient)
Alanine	2.7
Arginine	3.3
Aspartate	6.1
Cystine	0.25
Glutamate	19.6
Glycine	1.6
Histidine	2.5
Isoleucine	5.0
Leucine	8.0
Lysine	7.0
Methionine	2.5
Phenylalanine	4.4
Proline	9.2
Serine	4.8
Threonine	3.8
Tryptophan	1.0
Tyrosine	4.6
Valine	6.0

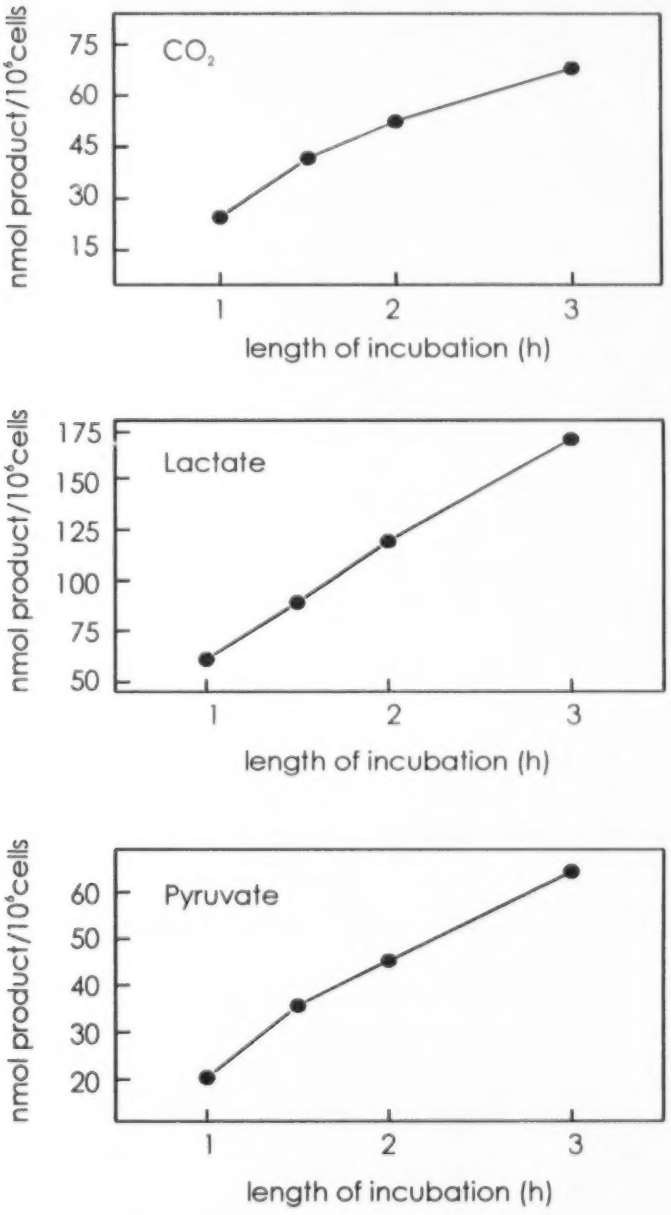
*Values listed are composite of assay data, textbook information, and extrapolation (Harlan Teklad, Madison WI).

Figure A-1: Dose response curves of splenocytes to mitogen stimulation



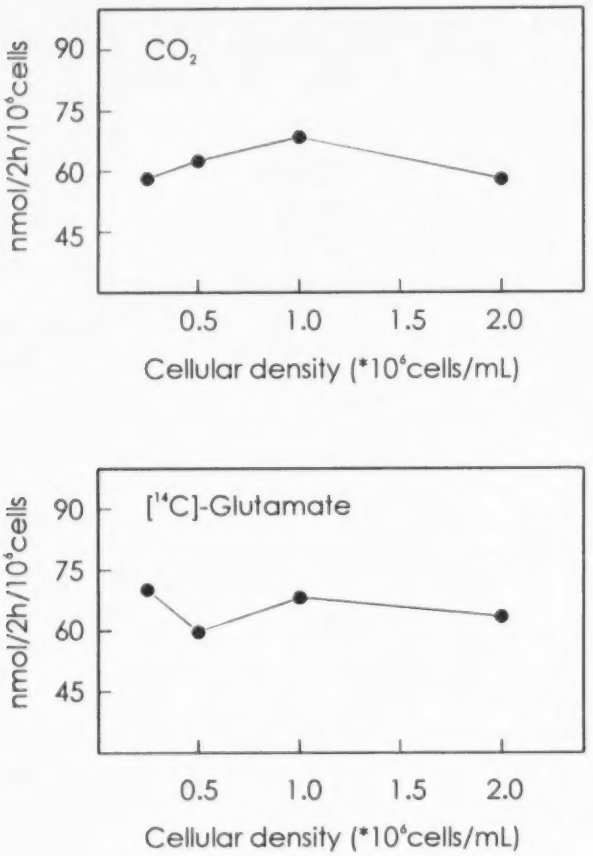
Each data point represents the mean of four determinations (n=1).
Cells were pulsed with 0.5 µCi [³H]-thymidine 18 hours prior to harvesting.
Arrow indicates optimum dose and length in culture.

Figure A-2: Time trials of glucose oxidation in freshly isolated Morris hepatoma 7777 cells (n=2)



Incubation media contained 4 mM [U-^{14}C] glucose. Incubation procedures are described in Materials and Methods, Chapter 5.

Figure A-3:
Cell concentration trials of glutamine oxidation in freshly isolated Morris hepatoma 7777 cells (n=2)



Incubation media contained 1 mM [U-^{14}C]-glutamine. Incubation conditions are described in Materials and Methods, Chapter 5.